

**PATENT APPLICATION
TRANSMITTAL**

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COMPOUNDS FOR IMMUNODIAGNOSIS OF PROSTATE CANCER AND METHODS FOR THEIR USE

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See MPEP chapter 600 concerning utility patent application contents.

Box Patent Application
Assistant Commissioner for
Washington, D.C. 20231

5. ☐ Incorporation By Reference (*useable if box 4b is checked*)
The entire disclosure of the prior application, from which a copy of the oath or declaration is supplied under Box 4b, is considered to be part of the disclosure of the accompanying application and is hereby incorporated by reference therein

6. ☐ Microfiche Computer Program (*Appendix*)
7. Nucleotide and Amino Acid Sequence Submission
(*if applicable, all necessary*)
- a. ☐ Computer-Readable Copy
- b. ☒ Paper Copy (identical to computer copy)
- c. ☐ Statement verifying identity of above copies

8. ☐ Assignment Papers (cover sheet & document(s))

9. ☐ 37 CFR 3.73(b) Statement ☐ Power of Attorney
(when there is an assignee)

10. ☐ English Translation Document (if applicable)

11. ☐ Information Disclosure Statement (IDS)/PTO-1449 ☐ Copies of IDS Citations

12. ☐ Preliminary Amendment

13. ☒ Return Receipt Postcard

14. ☐ Small Entity Statement(s) ☐ Statement filed in prior application, Status still proper and desired

15. ☐ Certified Copy of Priority Document(s)
(if foreign priority is claimed)

16. ☒ Other: Certificate of Express Mail

- 17. If a CONTINUING APPLICATION,** *check appropriate box and supply the requisite information below and in a preliminary amendment*

☐ Continuation ☐ Divisional ☒ Continuation-In-Part (CIP) of prior Application No.: **09/159,822**

Prior application information: Examiner _____ Group / Art Unit 1641

☐ Claims the benefit of Provisional Application No. _____

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REGISTRATION NO. 31,392

SIGNATURE 

Date JANUARY 15, 1999

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COMPOUNDS FOR IMMUNODIAGNOSIS OF PROSTATE CANCER
AND METHODS FOR THEIR USE

5 CROSS REFERENCE TO RELATED APPLICATIONS

This application is a continuation-in-part of U.S. Patent Application No. 09/159,822 filed September 23, 1998, which is a continuation-in-part of U.S. Patent Application No. 09/116,134, filed July 14, 1998, which is a continuation-in-part of U.S. Patent Application No. 09/030,606, filed February 25, 1998, which is a continuation-in-
10 part of U.S. Patent Application No. 09/020,747, filed February 9, 1998, which is a continuation-in-part of U.S. Patent Application No. 08/904,809, filed August 1, 1997, which is a continuation-in-part of U.S. Patent Application No. 08/806,596, filed February 25, 1997.

15 TECHNICAL FIELD

The present invention relates generally to cancer diagnosis and monitoring. The invention is more specifically related to polypeptides comprising at least a portion of a prostate tumor protein, and to polynucleotides encoding such polypeptides. Such polypeptides and polynucleotides may be used to generate
20 compounds for the diagnosis and monitoring of prostate cancer.

BACKGROUND OF THE INVENTION

Prostate cancer is the most common form of cancer among males, with an estimated incidence of 30% in men over the age of 50. Overwhelming clinical
25 evidence shows that human prostate cancer has the propensity to metastasize to bone, and the disease appears to progress inevitably from androgen dependent to androgen refractory status, leading to increased patient mortality. This prevalent disease is currently the second leading cause of cancer death among men in the U.S.

In spite of considerable research into therapies for the disease, prostate cancer remains difficult to treat. Commonly, treatment is based on surgery and/or radiation therapy, but these methods are ineffective in a significant percentage of cases. Two previously identified prostate specific proteins - prostate specific antigen (PSA) and prostatic acid phosphatase (PAP) - have limited therapeutic and diagnostic potential. For example, PSA levels do not always correlate well with the presence of prostate cancer, being positive in a percentage of non-prostate cancer cases, including benign prostatic hyperplasia (BPH). Furthermore, PSA measurements correlate with prostate volume, and do not indicate the level of metastasis.

In order to improve cancer treatment and survival, it would be beneficial to identify prostate tumor proteins that permit an earlier or more accurate diagnosis. In addition, further antigens are needed to facilitate the selection of a course of treatment and monitoring of patients. The present invention fulfills these needs and further provides other related advantages.

SUMMARY OF THE INVENTION

The present invention provides methods for diagnosis and monitoring of prostate cancer, together with kits for use in such methods. Polypeptides are disclosed which comprise at least an immunogenic portion of a prostate tumor protein or a variant thereof that differs in one or more substitutions, deletions, additions and/or insertions such that the ability of the variant to react with antigen-specific antisera is not substantially diminished. Within certain embodiments, the prostate tumor protein comprises an amino acid sequence encoded by a DNA molecule having a sequence selected from the group consisting of nucleotide sequences recited in SEQ ID NOs: 2-3, 5-107, 109-11, 115-171, 173-175, 177, 179-228, 229-305, 307-326, 328, 330, 332-335 and complements of such polynucleotides. Polynucleotides that encode all or a portion of a prostate tumor protein are also provided. Such polypeptides, polynucleotides, and compounds that bind to the polypeptides, may be used in the diagnosis and monitoring of cancer, such as prostate cancer.

In one specific aspect of the present invention, methods are provided for determining the presence or absence of prostate cancer in a patient, comprising: (a) contacting a biological sample obtained from a patient with a binding agent that is capable of binding to one of the above polypeptides; and (b) detecting in the sample an amount of polypeptide that binds to the binding agent, relative to a predetermined cut-off value, and therefrom determining the presence or absence of a cancer in the patient. In preferred embodiments, the binding agent is an antibody, most preferably a monoclonal antibody.

In related aspects, methods are provided for monitoring the progression of prostate cancer in a patient, comprising: (a) contacting a biological sample obtained from a patient with a binding agent that is capable of binding to one of the above polypeptides; (b) determining in the sample an amount of a protein or polypeptide that binds to the binding agent; (c) repeating steps (a) and (b); and comparing the amounts of polypeptide detected in steps (b) and (c).

Within related aspects, the present invention provides antibodies, preferably monoclonal antibodies, that bind to a polypeptide as described above, as well as diagnostic kits comprising such antibodies, and methods of using such antibodies to inhibit the development of prostate cancer.

The present invention further provides methods for determining the presence or absence of prostate cancer in a patient, comprising the steps of: (a) contacting a biological sample obtained from a patient with an oligonucleotide that hybridizes to a polynucleotide that encodes a prostate tumor protein, wherein the prostate tumor protein comprises an amino acid sequence that is encoded by a polynucleotide sequence selected from the group consisting of: (i) polynucleotides recited in any one of SEQ ID NOs:2-3, 5-107, 109-11, 115-171, 173-175, 177, 179-228, 229-305, 307-326, 328, 330, and 332-335; and (ii) complements of the foregoing polynucleotides; and (b) detecting in the sample a level of a polynucleotide that hybridizes to the oligonucleotide, relative to a predetermined cut-off value, and therefrom determining the presence or absence of prostate cancer in the patient. Within certain embodiments, the amount of mRNA is detected via polymerase chain reaction

using, for example, at least one oligonucleotide primer that hybridizes to a polynucleotide that encodes a polypeptide as recited above, or a complement of such a polynucleotide. Within other embodiments, the amount of mRNA is detected using a hybridization technique, employing an oligonucleotide probe that hybridizes to a polynucleotide that encodes a polypeptide as recited above, or a complement of such a polynucleotide. In a preferred embodiment, at least one of the oligonucleotide primers comprises at least about 10 contiguous nucleotides of a DNA molecule having a partial sequence selected from the group consisting of SEQ ID NOs:2-3, 5-107, 109-11, 115-171, 173-175, 177, 179-228, 229-305, 307-326, 328, 330, and 332-335.

10 In related aspects, methods are provided for monitoring the progression of prostate cancer in a patient, comprising the steps of: (a) contacting a biological sample obtained from a patient with an oligonucleotide that hybridizes to a polynucleotide that encodes a prostate tumor protein, wherein the antigen comprises an amino acid sequence that is encoded by a polynucleotide sequence selected from the group consisting of: (i) polynucleotides recited in any one of SEQ ID NOs:2-3, 5-107, 109-11, 115-171, 173-175, 177, 179-228, 229-305, 307-326, 328, 330, and 332-335; and (ii) complements of the foregoing polynucleotides; (b) detecting in the sample an amount of a polynucleotide that hybridizes to the oligonucleotide; (c) repeating steps (a) and (b) using a biological sample obtained from the patient at a subsequent point in time; and (d) comparing the amount of polynucleotide detected in step (c) with the amount detected in step (b) and therefrom monitoring the progression of prostate cancer in the patient.

In related aspects, diagnostic kits comprising the above oligonucleotide probes or primers are provided.

25 These and other aspects of the present invention will become apparent upon reference to the following detailed description and attached drawings. All references disclosed herein are hereby incorporated by reference in their entirety as if each was incorporated individually.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a graph illustrating the ability of T cells to kill fibroblasts expressing the representative prostate tumor polypeptide P502S, as compared to control fibroblasts. The % lysis is shown at a series of effector:target ratios, as indicated.

5 Figures 2A and 2B are graphs illustrating the ability of T cells to recognize cells expressing the representative prostate tumor polypeptide P502S. In each case, the number of γ -interferon spots is shown for different numbers of responders. In Figure 2A, data is presented for fibroblasts pulsed with the P2S-12 peptide, as compared to fibroblasts pulsed with a control E75 peptide. In Figure 2B, data is presented for fibroblasts expressing P506, as compared to fibroblasts expressing HER-
10 2/neu.

 Figure 3 represents a peptide competition binding assay showing that the P1S#10 peptide, derived from P501S, binds HLA-A2. Peptide P1S#10 inhibits HLA-A2 restricted presentation of fluM58 peptide to CTL clone D150M58 in TNF release
15 bioassay. D150M58 CTL is specific for the HLA-A2 binding influenza matrix peptide fluM58.

 Figure 4 is a graph illustrating the ability of T cell lines generated from P1S#10 immunized mice to specifically lyse P1S#10-pulsed Jurkat A2Kb targets and P501S-transduced Jurkat A2Kb targets, as compared to EGFP-transduced Jurkat A2Kb.
20 The per cent lysis is shown as a series of effector to target ratios, as indicated.

 Figures 5 illustrates the ability of a T cell clone to recognize and specifically lyse Jurkat A2Kb cells expressing the representative prostate tumor polypeptide P501S, thereby demonstrating that the P1S#10 peptide may be a naturally processed epitope of the P501S polypeptide.
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DETAILED DESCRIPTION OF THE INVENTION

As noted above, the present invention is generally directed to compounds and methods for the diagnosis and monitoring of prostate cancer. The compositions described herein may include one or more prostate tumor polypeptides, nucleic acid
30 sequences encoding such polypeptides, binding agents such as antibodies that bind to a polypeptide and/or immune system cells (*e.g.*, T cells). Prostate tumor polypeptides of

the present invention generally comprise at least a portion of a prostate tumor protein or a variant thereof, such that the therapeutic, antigenic and/or immunogenic properties of the polypeptide are not substantially diminished relative to the native prostate tumor protein. A "prostate tumor protein" is a protein that is overexpressed (*i.e.*, mRNA and/or protein is present at a level that is at least two fold higher) in prostate tumor tissue, relative to normal prostate tissue and/or relative to other tissues (*e.g.*, brain, heart, kidney, liver, lung, pancreas, ovary, placenta, skeletal muscle, spleen and/or thymus). Nucleic acid sequences of the subject invention generally comprise a DNA or RNA sequence that encodes all or a portion of such a polypeptide, or that is complementary to such a sequence. Antibodies are generally immune system proteins, or antigen-binding fragments thereof, that are capable of binding to a portion of a polypeptide as described above. T cells that may be employed within such compositions are generally T cells that are specific for a polypeptide as described above.

The present invention is based on the discovery of previously unknown human prostate tumor proteins. Partial sequences of polynucleotides encoding specific prostate tumor proteins (or complementary to such coding sequences) are provided in SEQ ID NOs:2-3, 5-107, 109-111, 115-171, 173-175, 177, 179-228, 229-305, 307-326, 328, 330, and 332-335.

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PROSTATE TUMOR POLYNUCLEOTIDES

The term "polynucleotide(s)," as used herein, means a single or double-stranded polymer of deoxyribonucleotide or ribonucleotide bases and includes DNA and corresponding RNA molecules, including HnRNA and mRNA molecules, both sense and anti-sense strands, and comprehends cDNA, genomic DNA and recombinant DNA, as well as wholly or partially synthesized polynucleotides. An HnRNA molecule contains introns and corresponds to a DNA molecule in a generally one-to-one manner. An mRNA molecule corresponds to an HnRNA and DNA molecule from which the introns have been excised. A polynucleotide may consist of an entire gene, or any portion thereof. Operable anti-sense polynucleotides may comprise a fragment of the

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corresponding polynucleotide, and the definition of "polynucleotide" therefore includes all such operable anti-sense fragments.

Any polynucleotide that encodes a prostate tumor protein or a portion or other variant thereof as described herein is encompassed by the present invention.

5 Preferred polynucleotides comprise at least 10 consecutive nucleotides, and preferably at least 30 consecutive nucleotides, that encode a portion of a prostate tumor protein. More preferably, a polynucleotide encodes an immunogenic portion of a prostate tumor protein. Polynucleotides complementary to any such sequences are also encompassed by the present invention.

10 Polynucleotides may comprise a native sequence (*i.e.*, an endogenous sequence that encodes a prostate tumor protein or a portion thereof) or may comprise a variant of such a sequence. Polynucleotide variants may contain one or more substitutions, additions, deletions and/or insertions such that the therapeutic, antigenic and/or immunogenic properties are not substantially diminished, relative to a native
15 prostate tumor protein. Such modifications may be readily introduced using standard mutagenesis techniques, such as oligonucleotide-directed site-specific mutagenesis as taught, for example, by Adelman et al. (*DNA*, 2:183, 1983). Preferably, the antigenicity or immunogenicity of a polypeptide variant is not substantially diminished. The effect on the immunogenicity of the encoded polypeptide may generally be assessed as
20 described herein. Variants preferably exhibit at least about 70% identity, more preferably at least about 80% identity and most preferably at least about 90% identity to a polynucleotide sequence that encodes a native prostate tumor protein or a portion thereof. The percent identity may be readily determined by comparing sequences using computer algorithms well known to those of ordinary skill in the art, such as Megalign,
25 using default parameters. Certain variants are substantially homologous to a native gene, or a portion or complement thereof. Such polynucleotide variants are capable of hybridizing under moderately stringent conditions to a naturally occurring DNA sequence encoding a native prostate tumor protein (or a complementary sequence). Suitable moderately stringent conditions include prewashing in a solution of 5 X SSC,
30 0.5% SDS, 1.0 mM EDTA (pH 8.0); hybridizing at 50°C-65°C, 5 X SSC, overnight;

followed by washing twice at 65°C for 20 minutes with each of 2X, 0.5X and 0.2X SSC containing 0.1% SDS).

It will be appreciated by those of ordinary skill in the art that, as a result of the degeneracy of the genetic code, there are many nucleotide sequences that encode a polypeptide as described herein. Some of these polynucleotides bear minimal homology to the nucleotide sequence of any native gene. Nonetheless, polynucleotides that vary due to differences in codon usage are specifically contemplated by the present invention.

Two nucleotide or polypeptide sequences are said to be “identical” if the sequence of nucleotides or amino acid residues in the two sequences is the same when aligned for maximum correspondence as described below. Comparisons between two sequences are typically performed by comparing the sequences over a comparison window to identify and compare local regions of sequence similarity. A “comparison window” as used herein, refers to a segment of at least about 20 contiguous positions, usually 30 to about 75, more preferably 40 to about 50, in which a sequence may be compared to a reference sequence of the same number of contiguous positions after the two sequences are optimally aligned.

Optimal alignment of sequences for comparison may be conducted using the Megalign program in the Lasergene suite of bioinformatics software (DNASTAR, Inc., Madison, WI), using default parameters. This program embodies several alignment schemes described in the following references: Dayhoff, M.O. (1978) A model of evolutionary change in proteins – Matrices for detecting distant relationships. In Dayhoff, M.O. (ed.) *Atlas of Protein Sequence and Structure*, National Biomedical Research Foundation, Washington DC Vol. 5, Suppl. 3, pp. 345-358; Hein J. (1990) Unified Approach to Alignment and Phylogenies pp. 626-645 *Methods in Enzymology* vol. 183, Academic Press, Inc., San Diego, CA; Higgins, D.G. and Sharp, P.M. (1989) Fast and sensitive multiple sequence alignments on a microcomputer *CABIOS* 5:151-153; Myers, E.W. and Muller W. (1988) Optimal alignments in linear space *CABIOS* 4:11-17; Robinson, E.D. (1971) *Comb. Theor* 11:105; Santou, N. Nes, M. (1987) The neighbor joining method. A new method for reconstructing phylogenetic trees *Mol.*

Biol. Evol. 4:406-425; Sneath, P.H.A. and Sokal, R.R. (1973) *Numerical Taxonomy – the Principles and Practice of Numerical Taxonomy*, Freeman Press, San Francisco, CA; Wilbur, W.J. and Lipman, D.J. (1983) Rapid similarity searches of nucleic acid and protein data banks *Proc. Natl. Acad. Sci. USA* 80:726-730.

5 Preferably, the “percentage of sequence identity” is determined by comparing two optimally aligned sequences over a window of comparison of at least 20 positions, wherein the portion of the polynucleotide sequence in the comparison window may comprise additions or deletions (i.e. gaps) of 20 percent or less, usually 5 to 15 percent, or 10 to 12 percent, as compared to the reference sequences (which does
10 not comprise additions or deletions) for optimal alignment of the two sequences. The percentage is calculated by determining the number of positions at which the identical nucleic acid bases or amino acid residue occurs in both sequences to yield the number of matched positions, dividing the number of matched positions by the total number of positions in the reference sequence (i.e. the window size) and multiplying the results by
15 100 to yield the percentage of sequence identity.

 Also included in the scope of the present invention are alleles of the genes encoding the nucleotide sequences recited herein. As used herein, an “allele” or “allelic sequence” is an alternative form of the gene which may result from at least one mutation in the nucleic acid sequence. Alleles may result in altered mRNAs or
20 polypeptides whose structure or function may or may not be altered. Any given gene may have none, one, or many allelic forms. Common mutational changes which give rise to alleles are generally ascribed to natural deletions, additions, or substitutions of nucleotides. Each of these types of changes may occur alone or in combination with the others, one or more times in a given sequence.

25 Polynucleotides may be prepared using any of a variety of techniques. For example, a polynucleotide may be identified, as described in more detail below, using a PCR-based subtraction protocol. Alternatively, polypeptides may be amplified via polymerase chain reaction (PCR) from cDNA prepared from prostate tumor cells. For this approach, sequence-specific primers may be designed based on the sequences
30 provided herein, and may be purchased or synthesized.

An amplified portion may be used to isolate a full length gene from a suitable library (e.g., a prostate tumor cDNA library) using well known techniques. Within such techniques, a library (cDNA or genomic) is screened using one or more polynucleotide probes or primers suitable for amplification. Preferably, a library is size-selected to include larger molecules. Random primed libraries may also be preferred for identifying 5' and upstream regions of genes. Genomic libraries are preferred for obtaining introns and extending 5' sequences.

For hybridization techniques, a partial sequence may be labeled (e.g., by nick-translation or end-labeling with ^{32}P) using well known techniques. A bacterial or bacteriophage library is then screened by hybridizing filters containing denatured bacterial colonies (or lawns containing phage plaques) with the labeled probe (see Sambrook et al., *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratories, Cold Spring Harbor, NY, 1989). Hybridizing colonies or plaques are selected and expanded, and the DNA is isolated for further analysis. cDNA clones may be analyzed to determine the amount of additional sequence by, for example, PCR using a primer from the partial sequence and a primer from the vector. Restriction maps and partial sequences may be generated to identify one or more overlapping clones. The complete sequence may then be determined using standard techniques, which may involve generating a series of deletion clones. The resulting overlapping sequences are then assembled into a single contiguous sequence. A full length cDNA molecule can be generated by ligating suitable fragments, using well known techniques.

Alternatively, there are numerous amplification techniques for obtaining a full length coding sequence from a partial cDNA sequence. Within such techniques, amplification is generally performed via PCR. Any of a variety of commercially available kits may be used to perform the amplification step. Primers may be designed using techniques well known in the art (see, for example, Mullis et al., *Cold Spring Harbor Symp. Quant. Biol.* 51:263, 1987; Erlich ed., *PCR Technology*, Stockton Press, NY, 1989), and software well known in the art may also be employed. Primers are preferably 22-30 nucleotides in length, have a GC content of at least 50% and anneal to the target sequence at temperatures of about 68°C to 72°C. The amplified region may

be sequenced as described above, and overlapping sequences assembled into a contiguous sequence.

One such amplification technique is inverse PCR (*see* Triglia et al., *Nucl. Acids Res.* 16:8186, 1988), which uses restriction enzymes to generate a fragment in the known region of the gene. The fragment is then circularized by intramolecular ligation and used as a template for PCR with divergent primers derived from the known region. Within an alternative approach, sequences adjacent to a partial sequence may be retrieved by amplification with a primer to a linker sequence and a primer specific to a known region. The amplified sequences are typically subjected to a second round of amplification with the same linker primer and a second primer specific to the known region. A variation on this procedure, which employs two primers that initiate extension in opposite directions from the known sequence, is described in WO 96/38591. Additional techniques include capture PCR (Lagerstrom et al., *PCR Methods Applic.* 1:111-19, 1991) and walking PCR (Parker et al., *Nucl. Acids. Res.* 19:3055-60, 1991). Transcription-Mediated Amplification, or TMA is another method that may be utilized for the amplification of DNA, rRNA, or mRNA, as described in Patent No. PCT/US91/03184. This autocatalytic and isothermic non-PCR based method utilizes two primers and two enzymes: RNA polymerase and reverse transcriptase. One primer contains a promoter sequence for RNA polymerase. In the first amplification, the promoter-primer hybridizes to the target rRNA at a defined site. Reverse transcriptase creates a DNA copy of the target rRNA by extension from the 3' end of the promoter-primer. The RNA in the resulting complex is degraded and a second primer binds to the DNA copy. A new strand of DNA is synthesized from the end of the primer by reverse transcriptase creating double stranded DNA. RNA polymerase recognizes the promoter sequence in the DNA template and initiates transcription. Each of the newly synthesized RNA amplicons re-enters the TMA process and serves as a template for a new round of replication leading to the exponential expansion of the RNA amplicon. Other methods employing amplification may also be employed to obtain a full length cDNA sequence.

In certain instances, it is possible to obtain a full length cDNA sequence

by analysis of sequences provided in an expressed sequence tag (EST) database, such as that available from GenBank. Searches for overlapping ESTs may generally be performed using well known programs (*e.g.*, NCBI BLAST searches), and such ESTs may be used to generate a contiguous full length sequence.

5 Certain nucleic acid sequences of cDNA molecules encoding portions of prostate tumor proteins are provided in SEQ ID NOS: 1-107, 109-111, 115-171, 173-175, 177, 179-228, 229-305, 307-326, 328, 330, and 332-335. The polynucleotides recited herein, as well as full length polynucleotides comprising such sequences, other portions of such full length polynucleotides, and sequences complementary to all or a
10 portion of such full length molecules, are specifically encompassed by the present invention.

 Polynucleotide variants may generally be prepared by any method known in the art, including chemical synthesis by, for example, solid phase phosphoramidite chemical synthesis. Modifications in a polynucleotide sequence may
15 also be introduced using standard mutagenesis techniques, such as oligonucleotide-directed site-specific mutagenesis (*see* Adelman et al., *DNA* 2:183, 1983). Alternatively, RNA molecules may be generated by *in vitro* or *in vivo* transcription of DNA sequences encoding a prostate tumor protein, or portion thereof, provided that the DNA is incorporated into a vector with a suitable RNA polymerase promoter (such as
20 T7 or SP6). Certain portions may be used to prepare an encoded polypeptide, as described herein. In addition, or alternatively, a portion may be administered to a patient such that the encoded polypeptide is generated *in vivo*.

 A portion of a sequence complementary to a coding sequence (*i.e.*, an antisense polynucleotide) may also be used as a probe or to modulate gene expression.
25 cDNA constructs that can be transcribed into antisense RNA may also be introduced into cells of tissues to facilitate the production of antisense RNA. An antisense polynucleotide may be used, as described herein, to inhibit expression of a prostate tumor protein. Antisense technology can be used to control gene expression through triple-helix formation, which compromises the ability of the double helix to open
30 sufficiently for the binding of polymerases, transcription factors or regulatory molecules

(see Gee et al., *In* Huber and Carr, *Molecular and Immunologic Approaches*, Futura Publishing Co. (Mt. Kisco, NY; 1994)). Alternatively, an antisense molecule may be designed to hybridize with a control region of a gene (*e.g.*, promoter, enhancer or transcription initiation site), and block transcription of the gene; or to block translation by inhibiting binding of a transcript to ribosomes.

A portion of a coding sequence or a complementary sequence may also be designed as a probe or primer to detect gene expression. Probes may be labeled by a variety of reporter groups, such as radionuclides and enzymes, and are preferably at least 10 nucleotides in length, more preferably at least 20 nucleotides in length and still more preferably at least 30 nucleotides in length. Primers, as noted above, are preferably 22-30 nucleotides in length.

Any polynucleotide may be further modified to increase stability *in vivo*. Possible modifications include, but are not limited to, the addition of flanking sequences at the 5' and/or 3' ends; the use of phosphorothioate or 2' O-methyl rather than phosphodiesterase linkages in the backbone; and/or the inclusion of nontraditional bases such as inosine, queosine and wybutosine, as well as acetyl- methyl-, thio- and other modified forms of adenine, cytidine, guanine, thymine and uridine.

Nucleotide sequences as described herein may be joined to a variety of other nucleotide sequences using established recombinant DNA techniques. For example, a polynucleotide may be cloned into any of a variety of cloning vectors, including plasmids, phagemids, lambda phage derivatives and cosmids. Vectors of particular interest include expression vectors, replication vectors, probe generation vectors and sequencing vectors. In general, a vector will contain an origin of replication functional in at least one organism, convenient restriction endonuclease sites and one or more selectable markers. Other elements will depend upon the desired use, and will be apparent to those of ordinary skill in the art.

Within certain embodiments, polynucleotides may be formulated so as to permit entry into a cell of a mammal, and expression therein. Such formulations are particularly useful for therapeutic purposes, as described below. Those of ordinary skill

in the art will appreciate that there are many ways to achieve expression of a polynucleotide in a target cell, and any suitable method may be employed. For example, a polynucleotide may be incorporated into a viral vector such as, but not limited to, adenovirus, adeno-associated virus, retrovirus, or vaccinia or other pox virus (e.g., avian pox virus). Techniques for incorporating polynucleotides into such vectors are well known to those of ordinary skill in the art. A retroviral vector may additionally transfer or incorporate a gene for a selectable marker (to aid in the identification or selection of transduced cells) and/or a targeting moiety, such as a gene that encodes a ligand for a receptor on a specific target cell, to render the vector target specific. Targeting may also be accomplished using an antibody, by methods known to those of ordinary skill in the art.

Other formulations for therapeutic purposes include colloidal dispersion systems, such as macromolecule complexes, nanocapsules, microspheres, beads, and lipid-based systems including oil-in-water emulsions, micelles, mixed micelles, and liposomes. A preferred colloidal system for use as a delivery vehicle *in vitro* and *in vivo* is a liposome (*i.e.*, an artificial membrane vesicle). The preparation and use of such systems is well known in the art.

PROSTATE TUMOR POLYPEPTIDES

Within the context of the present invention, polypeptides may comprise at least a portion of a prostate tumor protein or a variant thereof, as described herein. As noted above, a "prostate tumor protein" is a protein that is overexpressed by prostate tumor cells, relative to normal prostate cells and/or other tissues such as brain, heart, kidney, liver, lung, pancreas, ovary, placenta, skeletal muscle, spleen and/or thymus. Such polypeptides should comprise a portion of a prostate tumor protein such that the therapeutic, antigenic and/or immunogenic properties of the polypeptide are not substantially diminished, relative to the full length protein. Within certain preferred embodiments, a polypeptide comprises an immunogenic portion of a native prostate tumor protein (*i.e.*, the immunogenic properties of the polypeptide are not substantially diminished). As used herein, the term "polypeptide" encompasses amino acid chains of

any length, including full length proteins, wherein the amino acid residues are linked by covalent peptide bonds. In addition to a portion of a prostate tumor protein, additional sequences derived from the native protein and/or heterologous sequences may be present, and such sequences may (but need not) possess further immunogenic or antigenic properties.

An "immunogenic portion," as used herein is a portion of an antigen that is recognized (*i.e.*, specifically bound) by a B-cell and/or T-cell surface antigen receptor. Such immunogenic portions generally comprise at least 5 amino acid residues, more preferably at least 10, and still more preferably at least 20 amino acid residues of a prostate tumor protein or a variant thereof. Immunogenic portions of prostate tumor proteins provided herein may generally be identified using well known techniques, such as those summarized in Paul, *Fundamental Immunology*, 3rd ed., 243-247 (Raven Press, 1993) and references cited therein. Such techniques include screening polypeptides for the ability to react with antigen-specific antibodies, antisera and/or T-cell lines or clones. As used herein, antisera and antibodies are "antigen-specific" if they specifically bind to an antigen (*i.e.*, they react with the antigen in an ELISA or other immunoassay, and do not react detectably with unrelated proteins). Such antisera and antibodies may be prepared as described herein, and using well known techniques. An immunogenic portion of a native prostate tumor protein is a portion that reacts with such antisera and/or T-cells at a level that is not substantially less than the reactivity of the full length polypeptide (*e.g.*, in an ELISA and/or T-cell reactivity assay). Such immunogenic portions may react within such assays at a level that is similar to or greater than the reactivity of the full length polypeptide. Alternatively, an immunogenic portion may react within such assays at a level that is diminished by less than 50%, and preferably less than 20%, relative to the full length polypeptide. Such screens may generally be performed using methods well known to those of ordinary skill in the art, such as those described in Harlow and Lane, *Antibodies: A Laboratory Manual*, Cold Spring Harbor Laboratory, 1988. For example, a polypeptide may be immobilized on a solid support and contacted with patient sera to allow binding of antibodies within the sera to the immobilized polypeptide. Unbound

sera may then be removed and bound antibodies detected using, for example, ¹²⁵I-labeled Protein A.

As noted above, a polypeptide may comprise a variant of a native prostate tumor protein. A polypeptide "variant," as used herein, is a polypeptide that
5 differs from a native prostate tumor protein in one or more substitutions, deletions, additions and/or insertions, such that the therapeutic, antigenic and/or immunogenic properties are not substantially diminished. Preferably, the immunogenic properties are not substantially diminished. In other words, the ability of a variant to react with antigen-specific antisera may be enhanced or unchanged, relative to the native antigen,
10 or may be diminished by less than 50%, and preferably less than 20%, relative to the native antigen. Polypeptide variants preferably exhibit at least about 70%, more preferably at least about 90% and most preferably at least about 95% identity to polypeptides encoded by polynucleotides specifically recited herein. Identity may be determined by comparing sequences using computer algorithms well known to those of
15 skill in the art, such as Megalign, using default parameters. For prostate tumor polypeptides with immunoreactive properties, variants may generally be identified by modifying one of the above polypeptide sequences and evaluating the reactivity of the modified polypeptide with antigen-specific antibodies or antisera as described herein. For prostate tumor polypeptides useful for the generation of diagnostic binding agents, a
20 variant may be identified by evaluating a modified polypeptide for the ability to generate antibodies that detect the presence or absence of prostate cancer. Such modified sequences may be prepared and tested using, for example, the representative procedures described herein.

Preferably, a variant contains conservative substitutions. A
25 "conservative substitution" is one in which an amino acid is substituted for another amino acid that has similar properties, such that one skilled in the art of peptide chemistry would expect the secondary structure and hydrophobic nature of the polypeptide to be substantially unchanged. Amino acid substitutions may generally be made on the basis of similarity in polarity, charge, solubility, hydrophobicity,
30 hydrophilicity and/or the amphipathic nature of the residues. For example, negatively

charged amino acids include aspartic acid and glutamic acid; positively charged amino acids include lysine and arginine; and amino acids with uncharged polar head groups having similar hydrophilicity values include leucine, isoleucine and valine; glycine and alanine; asparagine and glutamine; and serine, threonine, phenylalanine and tyrosine.

5 Other groups of amino acids that may represent conservative changes include: (1) ala, pro, gly, glu, asp, gln, asn, ser, thr; (2) cys, ser, tyr, thr; (3) val, ile, leu, met, ala, phe; (4) lys, arg, his; and (5) phe, tyr, trp, his. A variant may also, or alternatively, contain nonconservative changes. Variants containing substitutions may also (or alternatively) be modified by, for example, the deletion or addition of amino acids that have minimal
10 influence on the immunogenicity, secondary structure and hydrophobic nature of the polypeptide.

As noted above, polypeptides may comprise a signal (or leader) sequence at the N-terminal end of the protein which co-translationally or post-translationally directs transfer of the protein. The polypeptide may also be conjugated
15 to a linker or other sequence for ease of synthesis, purification or identification of the polypeptide (*e.g.*, poly-His), or to enhance binding of the polypeptide to a solid support. For example, a polypeptide may be conjugated to an immunoglobulin Fc region.

Polypeptides may be prepared using any of a variety of well known techniques. Recombinant polypeptides encoded by polynucleotide sequences as described above may be readily prepared from the polynucleotide sequences using any
20 of a variety of expression vectors known to those of ordinary skill in the art. Expression may be achieved in any appropriate host cell that has been transformed or transfected with an expression vector containing a polynucleotide molecule that encodes a recombinant polypeptide. Suitable host cells include prokaryotes, yeast and higher
25 eukaryotic cells. Preferably, the host cells employed are *E. coli*, yeast or a mammalian cell line, such as CHO cells. Supernatants from suitable host/vector systems which secrete recombinant protein or polypeptide into culture media may be first concentrated using a commercially available filter. Following concentration, the concentrate may be applied to a suitable purification matrix such as an affinity matrix or an ion exchange
30 resin. Finally, one or more reverse phase HPLC steps can be employed to further purify

a recombinant polypeptide.

Portions and other variants having fewer than about 100 amino acids, and generally fewer than about 50 amino acids, may also be generated by synthetic means, using techniques well known to those of ordinary skill in the art. For example, such polypeptides may be synthesized using any of the commercially available solid-phase techniques, such as the Merrifield solid-phase synthesis method, where amino acids are sequentially added to a growing amino acid chain. *See* Merrifield, *J. Am. Chem. Soc.* 85:2149-2146, 1963. Equipment for automated synthesis of polypeptides is commercially available from suppliers such as Applied BioSystems, Inc. (Foster City, CA), and may be operated according to the manufacturer's instructions.

Within certain specific embodiments, a polypeptide may be a fusion protein that comprises multiple polypeptides as described herein, or that comprises one polypeptide as described herein and a known prostate tumor antigen, or a variant of such an antigen. A fusion protein generally comprises at least one of the above immunogenic portions and one or more additional immunogenic prostate tumor sequences, which are joined via a peptide linkage into a single amino acid chain. The sequences may be joined directly (*i.e.*, with no intervening amino acids) or may be joined by way of a linked sequence (*e.g.*, Gly-Cys-Gly) that does not significantly diminish the immunogenic properties of the component polypeptides.

Fusion proteins may generally be prepared using standard techniques. For example, a fusion protein may be prepared recombinantly. Briefly, DNA sequences encoding the polypeptide components may be assembled separately, and ligated into an appropriate expression vector. The 3' end of the DNA sequence encoding one polypeptide component is ligated, with or without a peptide linker, to the 5' end of a DNA sequence encoding the second polypeptide component so that the reading frames of the sequences are in phase. This permits translation into a single fusion protein that retains the biological activity of both component polypeptides.

A peptide linker sequence may be employed to separate the first and the second polypeptide components by a distance sufficient to ensure that each polypeptide folds into its secondary and tertiary structures. Such a peptide linker sequence may be

incorporated into the fusion protein using standard techniques well known in the art. Suitable peptide linker sequences may be chosen based on the following factors: (1) their ability to adopt a flexible extended conformation; (2) their inability to adopt a secondary structure that could interact with functional epitopes on the first and second polypeptides; and (3) the lack of hydrophobic or charged residues that might react with the polypeptide functional epitopes. Preferred peptide linker sequences contain Gly, Asn and Ser residues. Other near neutral amino acids, such as Thr and Ala may also be used in the linker sequence. Amino acid sequences which may be usefully employed as linkers include those disclosed in Maratea et al., *Gene* 40:39-46, 1985; Murphy et al., *Proc. Natl. Acad. Sci. USA* 83:8258-8262, 1986; U.S. Patent No. 4,935,233 and U.S. Patent No. 4,751,180. The linker sequence may generally be from 1 to about 50 amino acids in length. Linker sequences are not required when the first and second polypeptides have non-essential N-terminal amino acid regions that can be used to separate the functional domains and prevent steric interference.

The ligated polynucleotide sequences are operably linked to suitable transcriptional or translational regulatory elements. The regulatory elements responsible for expression of polynucleotide are located only 5' to the polynucleotide sequence encoding the first polypeptides. Similarly, stop codons required to end translation and transcription termination signals are only present 3' to the polynucleotide sequence encoding the second polypeptide.

Fusion proteins are also provided that comprise a polypeptide of the present invention together with an unrelated immunogenic protein. Preferably the immunogenic protein is capable of eliciting a recall response. Examples of such proteins include tetanus, tuberculosis and hepatitis proteins (*see*, for example, Stoute et al. *New Engl. J. Med.*, 336:86-91, 1997).

In general, polypeptides (including fusion proteins) and polynucleotides as described herein are isolated. An "isolated" polypeptide or polynucleotide is one that is removed from its original environment. For example, a naturally-occurring protein is isolated if it is separated from some or all of the coexisting materials in the natural system. Preferably, such polypeptides are at least about 90% pure, more preferably at

least about 95% pure and most preferably at least about 99% pure. A polynucleotide is considered to be isolated if, for example, it is cloned into a vector that is not a part of the natural environment.

5 BINDING AGENTS

The present invention further provides agents, such as antibodies and antigen-binding fragments thereof, that specifically bind to a prostate tumor protein. As used herein, an agent is said to "specifically bind" to a prostate tumor protein if it reacts at a detectable level (within, for example, an ELISA) with a prostate tumor protein, and
10 does not react detectably with unrelated proteins under similar conditions. As used herein, "binding" refers to a noncovalent association between two separate molecules such that a "complex" is formed. The ability to bind may be evaluated by, for example, determining a binding constant for the formation of the complex. The binding constant is the value obtained when the concentration of the complex is divided by the product of
15 the component concentrations. In general, two compounds are said to "bind," in the context of the present invention, when the binding constant for complex formation exceeds about 10^3 L/mol. The binding constant may be determined using methods well known in the art.

Binding agents are further capable of detecting metastatic prostate
20 tumors and differentiating between patients with and without prostate cancer, using a representative assay provided herein. In other words, antibodies or other binding agents that bind to a prostate tumor protein will generate a signal indicating the presence of prostate cancer in at least about 20% of patients with the disease, and will generate a negative signal indicating the absence of the disease in at least about 90% of individuals
25 without the cancer. To determine whether a binding agent satisfies this requirement, biological samples (*e.g.*, blood, blood-associated tumor cells, sera, urine, biopsies and/or prostate secretions) from patients with and without prostate cancer (as determined using standard clinical tests) may be assayed as described herein for the presence of polypeptides or polynucleotides that bind to the binding agent. It will be
30 apparent that a statistically significant number of samples with and without the disease

should be assayed. Each binding agent should satisfy the above criteria; however, those of ordinary skill in the art will recognize that binding agents may be used in combination to improve sensitivity.

If an immunogenic portion is employed, the resulting antibody should
5 indicate the presence of prostate cancer in substantially all (*i.e.*, at least 80%, and preferably at least 90%) of the patients for which prostate cancer would be indicated using an antibody raised against the full length antigen. The antibody should also indicate the absence of prostate cancer in substantially all of those samples that would be negative when tested with an antibody raised against the full length antigen. The
10 representative assays provided herein, such as the two-antibody sandwich assay, may generally be employed for evaluating the ability of an antibody to detect prostate cancer.

Binding agents may be further linked to a reporter group, to facilitate diagnostic assays. Suitable reporter groups will be apparent to those of ordinary skill in
15 the art, and include enzymes (such as horseradish peroxidase), substrates, cofactors, inhibitors, dyes, radionuclides, luminescent groups, fluorescent groups and biotin. The conjugation of antibody to reporter group may be achieved using standard methods known to those of ordinary skill in the art.

Any agent that satisfies the above requirements may be a binding agent.
20 For example, a binding agent may be a ribosome, with or without a peptide component, an RNA molecule or a polypeptide. In a preferred embodiment, a binding agent is an antibody or an antigen-binding fragment thereof. Such antibodies may be polyclonal or monoclonal. In addition, the antibodies may be single chain, chimeric, CDR-grafted or humanized.

25 Antibodies may be prepared by any of a variety of techniques known to those of ordinary skill in the art. *See, e.g.*, Harlow and Lane, *Antibodies: A Laboratory Manual*, Cold Spring Harbor Laboratory, 1988. In general, antibodies can be produced by cell culture techniques, including the generation of monoclonal antibodies as described herein, or via transfection of antibody genes into suitable bacterial or
30 mammalian cell hosts, in order to allow for the production of recombinant antibodies.

In one technique, an immunogen comprising the polypeptide is initially injected into any of a wide variety of mammals (*e.g.*, mice, rats, rabbits, sheep or goats). In this step, the polypeptides of this invention may serve as the immunogen without modification. Alternatively, particularly for relatively short polypeptides, a superior immune response may be elicited if the polypeptide is joined to a carrier protein, such as bovine serum albumin or keyhole limpet hemocyanin. The immunogen is injected into the animal host, preferably according to a predetermined schedule incorporating one or more booster immunizations, and the animals are bled periodically. Polyclonal antibodies specific for the polypeptide may then be purified from such antisera by, for example, affinity chromatography using the polypeptide coupled to a suitable solid support.

Monoclonal antibodies specific for the antigenic polypeptide of interest may be prepared, for example, using the technique of Kohler and Milstein, *Eur. J. Immunol.* 6:511-519, 1976, and improvements thereto. Briefly, these methods involve the preparation of immortal cell lines capable of producing antibodies having the desired specificity (*i.e.*, reactivity with the polypeptide of interest). Such cell lines may be produced, for example, from spleen cells obtained from an animal immunized as described above. The spleen cells are then immortalized by, for example, fusion with a myeloma cell fusion partner, preferably one that is syngeneic with the immunized animal. A variety of fusion techniques may be employed. For example, the spleen cells and myeloma cells may be combined with a nonionic detergent for a few minutes and then plated at low density on a selective medium that supports the growth of hybrid cells, but not myeloma cells. A preferred selection technique uses HAT (hypoxanthine, aminopterin, thymidine) selection. After a sufficient time, usually about 1 to 2 weeks, colonies of hybrids are observed. Single colonies are selected and their culture supernatants tested for binding activity against the polypeptide. Hybridomas having high reactivity and specificity are preferred.

Monoclonal antibodies may be isolated from the supernatants of growing hybridoma colonies. In addition, various techniques may be employed to enhance the yield, such as injection of the hybridoma cell line into the peritoneal cavity of a suitable vertebrate host, such as a mouse. Monoclonal antibodies may then be harvested from

the ascites fluid or the blood. Contaminants may be removed from the antibodies by conventional techniques, such as chromatography, gel filtration, precipitation, and extraction. The polypeptides of this invention may be used in the purification process within, for example, an affinity chromatography step.

5 Within certain embodiments, the use of antigen-binding fragments of antibodies may be preferred. Such fragments include Fab fragments, which may be prepared using standard techniques. Briefly, immunoglobulins may be purified from rabbit serum by affinity chromatography on Protein A bead columns (Harlow and Lane, *Antibodies: A Laboratory Manual*, Cold Spring Harbor Laboratory, 1988) and digested
10 by papain to yield Fab and Fc fragments. The Fab and Fc fragments may be separated by affinity chromatography on protein A bead columns.

Monoclonal antibodies of the present invention may be coupled to one or more therapeutic agents. Suitable agents in this regard include radionuclides, differentiation inducers, drugs, toxins, and derivatives thereof. Preferred radionuclides
15 include ^{90}Y , ^{123}I , ^{125}I , ^{131}I , ^{186}Re , ^{188}Re , ^{211}At , and ^{212}Bi . Preferred drugs include methotrexate, and pyrimidine and purine analogs. Preferred differentiation inducers include phorbol esters and butyric acid. Preferred toxins include ricin, abrin, diphtheria toxin, cholera toxin, gelonin, *Pseudomonas* exotoxin, *Shigella* toxin, and pokeweed antiviral protein.

20 A therapeutic agent may be coupled (*e.g.*, covalently bonded) to a suitable monoclonal antibody either directly or indirectly (*e.g.*, via a linker group). A direct reaction between an agent and an antibody is possible when each possesses a substituent capable of reacting with the other. For example, a nucleophilic group, such as an amino or sulfhydryl group, on one may be capable of reacting with a carbonyl-
25 containing group, such as an anhydride or an acid halide, or with an alkyl group containing a good leaving group (*e.g.*, a halide) on the other.

Alternatively, it may be desirable to couple a therapeutic agent and an antibody via a linker group. A linker group can function as a spacer to distance an antibody from an agent in order to avoid interference with binding capabilities. A
30 linker group can also serve to increase the chemical reactivity of a substituent on an

agent or an antibody, and thus increase the coupling efficiency. An increase in chemical reactivity may also facilitate the use of agents, or functional groups on agents, which otherwise would not be possible.

It will be evident to those skilled in the art that a variety of bifunctional or polyfunctional reagents, both homo- and hetero-functional (such as those described in the catalog of the Pierce Chemical Co., Rockford, IL), may be employed as the linker group. Coupling may be effected, for example, through amino groups, carboxyl groups, sulfhydryl groups or oxidized carbohydrate residues. There are numerous references describing such methodology, *e.g.*, U.S. Patent No. 4,671,958, to Rodwell et al.

Where a therapeutic agent is more potent when free from the antibody portion of the immunoconjugates of the present invention, it may be desirable to use a linker group which is cleavable during or upon internalization into a cell. A number of different cleavable linker groups have been described. The mechanisms for the intracellular release of an agent from these linker groups include cleavage by reduction of a disulfide bond (*e.g.*, U.S. Patent No. 4,489,710, to Spitler), by irradiation of a photolabile bond (*e.g.*, U.S. Patent No. 4,625,014, to Senter et al.), by hydrolysis of derivatized amino acid side chains (*e.g.*, U.S. Patent No. 4,638,045, to Kohn et al.), by serum complement-mediated hydrolysis (*e.g.*, U.S. Patent No. 4,671,958, to Rodwell et al.), and acid-catalyzed hydrolysis (*e.g.*, U.S. Patent No. 4,569,789, to Blattler et al.).

It may be desirable to couple more than one agent to an antibody. In one embodiment, multiple molecules of an agent are coupled to one antibody molecule. In another embodiment, more than one type of agent may be coupled to one antibody. Regardless of the particular embodiment, immunoconjugates with more than one agent may be prepared in a variety of ways. For example, more than one agent may be coupled directly to an antibody molecule, or linkers which provide multiple sites for attachment can be used. Alternatively, a carrier can be used.

A carrier may bear the agents in a variety of ways, including covalent bonding either directly or via a linker group. Suitable carriers include proteins such as albumins (*e.g.*, U.S. Patent No. 4,507,234, to Kato et al.), peptides and polysaccharides such as aminodextran (*e.g.*, U.S. Patent No. 4,699,784, to Shih et al.). A carrier may

also bear an agent by noncovalent bonding or by encapsulation, such as within a liposome vesicle (*e.g.*, U.S. Patent Nos. 4,429,008 and 4,873,088). Carriers specific for radionuclide agents include radiohalogenated small molecules and chelating compounds. For example, U.S. Patent No. 4,735,792 discloses representative
5 radiohalogenated small molecules and their synthesis. A radionuclide chelate may be formed from chelating compounds that include those containing nitrogen and sulfur atoms as the donor atoms for binding the metal, or metal oxide, radionuclide. U.S. Patent No. 4,673,562, to Davison et al. discloses representative chelating compounds and their synthesis.

10 A variety of routes of administration for the antibodies and immunoconjugates may be used. Typically, administration is intravenous, intramuscular, subcutaneous or in the bed of a resected tumor. It will be evident that the precise dose of the antibody/immunoconjugate will vary depending upon the antibody used, the antigen density on the tumor, and the rate of clearance of the antibody.

15

PHARMACEUTICAL COMPOSITIONS AND VACCINES

Within certain aspects, polypeptides, polynucleotides and/or binding agents may be incorporated into pharmaceutical compositions or vaccines. Pharmaceutical compositions comprise one or more such compounds and a
20 physiologically acceptable carrier. Vaccines may comprise one or more such compounds and a non-specific immune response enhancer. A non-specific immune response enhancer may be any substance that enhances an immune response to an exogenous antigen. Examples of non-specific immune response enhancers include adjuvants, biodegradable microspheres (*e.g.*, polylactic galactide) and liposomes (into
25 which the compound is incorporated). Pharmaceutical compositions and vaccines within the scope of the present invention may also contain other compounds, which may be biologically active or inactive. For example, one or more immunogenic portions of other tumor antigens may be present, either incorporated into a fusion polypeptide or as a separate compound within the composition or vaccine.

30

A pharmaceutical composition or vaccine may contain polynucleotides

encoding one or more of the polypeptides as described above, such that the polypeptide is generated *in situ*. As noted above, the polynucleotides may be present within any of a variety of delivery systems known to those of ordinary skill in the art, including nucleic acid expression systems, bacteria and viral expression systems. Appropriate nucleic acid expression systems contain the necessary polynucleotide sequences for expression in the patient (such as a suitable promoter and terminating signal). Bacterial delivery systems involve the administration of a bacterium (such as *Bacillus-Calmette-Guerrin*) that expresses an immunogenic portion of the polypeptide on its cell surface. In a preferred embodiment, the polynucleotides may be introduced using a viral expression system (e.g., vaccinia or other pox virus, retrovirus, or adenovirus), which may involve the use of a non-pathogenic (defective), replication competent virus. Suitable systems are disclosed, for example, in Fisher-Hoch et al., *PNAS* 86:317-321, 1989; Flexner et al., *Ann. N.Y. Acad. Sci.* 569:86-103, 1989; Flexner et al., *Vaccine* 8:17-21, 1990; U.S. Patent Nos. 4,603,112, 4,769,330, and 5,017,487; WO 89/01973; U.S. Patent No. 4,777,127; GB 2,200,651; EP 0,345,242; WO 91/02805; Berkner, *Biotechniques* 6:616-627, 1988; Rosenfeld et al., *Science* 252:431-434, 1991; Kolls et al., *PNAS* 91:215-219, 1994; Kass-Eisler et al., *PNAS* 90:11498-11502, 1993; Guzman et al., *Circulation* 88:2838-2848, 1993; and Guzman et al., *Cir. Res.* 73:1202-1207, 1993. Techniques for incorporating polynucleotides into such expression systems are well known to those of ordinary skill in the art. The polynucleotides may also be "naked," as described, for example, in Ulmer et al., *Science* 259:1745-1749, 1993 and reviewed by Cohen, *Science* 259:1691-1692, 1993. The uptake of naked polynucleotides may be increased by coating the polynucleotides onto biodegradable beads, which are efficiently transported into the cells.

While any suitable carrier known to those of ordinary skill in the art may be employed in the pharmaceutical compositions of this invention, the type of carrier will vary depending on the mode of administration. Compositions of the present invention may be formulated for any appropriate manner of administration including, for example, topical, oral, nasal, intravenous, intracranial, intraperitoneal, subcutaneous or intramuscular administration. For parenteral administration, such as subcutaneous

injection, the carrier preferably comprises water, saline, alcohol, a fat, a wax or a buffer. For oral administration, any of the above carriers or a solid carrier, such as mannitol, lactose, starch, magnesium stearate, sodium saccharine, talcum, cellulose, glucose, sucrose, and magnesium carbonate, may be employed. Biodegradable microspheres
5 (e.g., polylactate polyglycolate) may also be employed as carriers for the pharmaceutical compositions of this invention. Suitable biodegradable microspheres are disclosed, for example, in U.S. Patent Nos. 4,897,268 and 5,075,109.

Such compositions may also comprise buffers (e.g., neutral buffered saline or phosphate buffered saline), carbohydrates (e.g., glucose, mannose, sucrose or
10 dextrans), mannitol, proteins, polypeptides or amino acids such as glycine, antioxidants, chelating agents such as EDTA or glutathione, adjuvants (e.g., aluminum hydroxide) and/or preservatives. Alternatively, compositions of the present invention may be formulated as a lyophilizate. Compounds may also be encapsulated within liposomes using well known technology.

15 Any of a variety of non-specific immune response enhancers may be employed in the vaccines of this invention. For example, an adjuvant may be included. Most adjuvants contain a substance designed to protect the antigen from rapid catabolism, such as aluminum hydroxide or mineral oil, and a stimulator of immune responses, such as lipid A, *Bordetella pertussis* or *Mycobacterium tuberculosis* derived
20 proteins. Suitable adjuvants are commercially available as, for example, Freund's Incomplete Adjuvant and Complete Adjuvant (Difco Laboratories, Detroit, MI), Merck Adjuvant 65 (Merck and Company, Inc., Rahway, NJ), alum, biodegradable microspheres, monophosphoryl lipid A and quil A. Cytokines, such as GM-CSF or interleukin-2, -7, or -12, may also be used as adjuvants.

25 The compositions described herein may be administered as part of a sustained release formulation (i.e., a formulation such as a capsule or sponge that effects a slow release of compound following administration). Such formulations may generally be prepared using well known technology and administered by, for example, oral, rectal or subcutaneous implantation, or by implantation at the desired target site.
30 Sustained-release formulations may contain a polypeptide, polynucleotide or antibody

dispersed in a carrier matrix and/or contained within a reservoir surrounded by a rate controlling membrane. Carriers for use within such formulations are biocompatible, and may also be biodegradable; preferably the formulation provides a relatively constant level of active component release. The amount of active compound contained
5 within a sustained release formulation depends upon the site of implantation, the rate and expected duration of release and the nature of the condition to be treated or prevented.

CANCER THERAPY

10 In further aspects of the present invention, the pharmaceutical compositions and vaccines described herein may be used for immunotherapy of cancer, such as prostate cancer, in a patient. Polypeptides for use within such compositions and vaccines generally comprise an immunogenic portion of a prostate tumor protein, or a variant thereof. Such polypeptides may stimulate the patient's own immune response to
15 prostate tumor cells. Alternatively, a pharmaceutical composition or vaccine may comprise one or more fusion proteins comprising one or more such polypeptides and/or polynucleotides encoding such one or more such polypeptides. Monoclonal antibodies of the present invention may also be used as therapeutic reagents, to diminish or eliminate prostate tumors. The antibodies may be used on their own (for instance, to
20 inhibit metastases) or coupled to one or more therapeutic agents, as described above.

Within such methods, pharmaceutical compositions and vaccines are typically administered to a patient. As used herein, a "patient" refers to any warm-blooded animal, preferably a human. A patient may be afflicted with a disease, or may be free of detectable disease. Accordingly, the above pharmaceutical compositions and
25 vaccines may be used to prevent the development of prostate cancer or to treat a patient afflicted with prostate cancer. Prostate cancer may be diagnosed using criteria generally accepted in the art. Pharmaceutical compositions and vaccines may be administered either prior to or following surgical removal of primary tumors and/or treatment such as administration of radiotherapy or conventional chemotherapeutic drugs.

30 Routes and frequency of administration, as well as dosage, will vary

from individual to individual, and may parallel those currently being used in immunotherapy of other diseases. In general, the pharmaceutical compositions and vaccines may be administered by injection (*e.g.*, intracutaneous, intramuscular, intravenous or subcutaneous), intranasally (*e.g.*, by aspiration) or orally. Preferably, between 1 and 10 doses may be administered over a 3-24 week period. Preferably, 4 doses are administered, at an interval of 3 months, and booster administrations may be given periodically thereafter. Alternate protocols may be appropriate for individual patients. A suitable dose is an amount of polypeptide or polynucleotide that is effective to raise an immune response (cellular and/or humoral) against prostate tumor cells in a treated patient. A suitable immune response is at least 10-50% above the basal (*i.e.*, untreated) level. Such response can be monitored by measuring the anti-tumor antibodies in a patient or by vaccine-dependent generation of cytolytic effector cells capable of killing the patient's tumor cells *in vitro*. Such vaccines should also be capable of causing an immune response that leads to an improved clinical outcome (*e.g.*, more frequent remissions, complete or partial or longer disease-free survival) in vaccinated patients as compared to non-vaccinated patients. In general, the amount of polypeptide present in a dose (or produced *in situ* by the polynucleotides molecule in a dose) ranges from about 1 pg to about 100 mg per kg of host, typically from about 10 pg to about 1 mg, and preferably from about 100 pg to about 1 μ g. Suitable dose sizes will vary with the size of the patient, but will typically range from about 0.01 mL to about 5 mL. A variety of routes of administration for the antibodies and immunoconjugates may be used. Typically, administration will be intravenous, intramuscular, subcutaneous or in the bed of a resected tumor. It will be evident that the precise dose of the antibody/immunoconjugate will vary depending upon the antibody used, the antigen density on the tumor, and the rate of clearance of the antibody.

Polypeptides disclosed herein may also be employed in adoptive immunotherapy for the treatment of cancer. Adoptive immunotherapy may be broadly classified into either active or passive immunotherapy. In active immunotherapy, treatment relies on the *in vivo* stimulation of the endogenous host immune system to

react against tumors with the administration of immune response-modifying agents (for example, tumor vaccines, bacterial adjuvants, and/or cytokines).

In passive immunotherapy, treatment involves the delivery of biologic reagents with established tumor-immune reactivity (such as effector cells or antibodies) that can directly or indirectly mediate antitumor effects and does not necessarily depend on an intact host immune system. Examples of effector cells include T lymphocytes (for example, CD8⁺ cytotoxic T-lymphocyte, CD4⁺ T-helper, gamma/delta T lymphocytes, tumor-infiltrating lymphocytes), killer cells (such as Natural Killer cells, lymphokine-activated killer cells), B cells, or antigen presenting cells (such as dendritic cells and macrophages) expressing the disclosed antigens. The polypeptides disclosed herein may also be used to generate antibodies or anti-idiotypic antibodies (as in U.S. Patent No. 4,918,164), for passive immunotherapy.

The predominant method of procuring adequate numbers of T-cells for adoptive immunotherapy is to grow immune T-cells *in vitro*. Culture conditions for expanding single antigen-specific T-cells to several billion in number with retention of antigen recognition *in vivo* are well known in the art. These *in vitro* culture conditions typically utilize intermittent stimulation with antigen, often in the presence of cytokines, such as IL-2, and non-dividing feeder cells. As noted above, the immunoreactive polypeptides described herein may be used to rapidly expand antigen-specific T cell cultures in order to generate sufficient number of cells for immunotherapy. In particular, antigen-presenting cells, such as dendritic, macrophage, monocyte, fibroblast, or B-cells, may be pulsed with immunoreactive polypeptides, or polynucleotide sequence(s) may be introduced into antigen presenting cells, using a variety of standard techniques well known in the art. For example, antigen presenting cells may be transfected or transduced with a polynucleotide sequence, wherein said sequence contains a promoter region appropriate for increasing expression, and can be expressed as part of a recombinant virus or other expression system. Several viral vectors may be used to transduce an antigen presenting cell, including pox virus, vaccinia virus, and adenovirus; also, antigen presenting cells may be transfected with polynucleotide sequences disclosed herein by a variety of means, including gene-gun

technology, lipid-mediated delivery, electroporation, osmotic shock, and particulate delivery mechanisms, resulting in efficient and acceptable expression levels as determined by one of ordinary skill in the art. For cultured T-cells to be effective in therapy, the cultured T-cells must be able to grow and distribute widely and to survive
5 long term *in vivo*. Studies have demonstrated that cultured T-cells can be induced to grow *in vivo* and to survive long term in substantial numbers by repeated stimulation with antigen supplemented with IL-2 (see, for example, Cheever, M., *et al*, "Therapy With Cultured T Cells: Principles Revisited," *Immunological Reviews*, 157:177, 1997).

The polypeptides disclosed herein may also be employed to generate
10 and/or isolate tumor-reactive T-cells, which can then be administered to the patient. In one technique, antigen-specific T-cell lines may be generated by *in vivo* immunization with short peptides corresponding to immunogenic portions of the disclosed polypeptides. The resulting antigen specific CD8+ CTL clones may be isolated from the patient, expanded using standard tissue culture techniques, and returned to the
15 patient.

Alternatively, peptides corresponding to immunogenic portions of the polypeptides may be employed to generate tumor reactive T cell subsets by selective *in vitro* stimulation and expansion of autologous T cells to provide antigen-specific T cells which may be subsequently transferred to the patient as described, for example, by
20 Chang *et al*, (*Crit. Rev. Oncol. Hematol.*, 22(3), 213, 1996). Cells of the immune system, such as T cells, may be isolated from the peripheral blood of a patient, using a commercially available cell separation system, such as CellPro Incorporated's (Bothell, WA) CEPRATE™ system (see U.S. Patent No. 5,240,856; U.S. Patent No. 5,215,926; WO 89/06280; WO 91/16116 and WO 92/07243). The separated cells are stimulated
25 with one or more of the immunoreactive polypeptides contained within a delivery vehicle, such as a microsphere, to provide antigen-specific T cells. The population of tumor antigen-specific T cells is then expanded using standard techniques and the cells are administered back to the patient.

In other embodiments, T-cell and/or antibody receptors specific for the
30 polypeptides disclosed herein can be cloned, expanded, and transferred into other

vectors or effector cells for use in adoptive immunotherapy. In particular, T cells may be transfected with the appropriate genes to express the variable domains from tumor specific monoclonal antibodies as the extracellular recognition elements and joined to the T cell receptor signaling chains, resulting in T cell activation, specific lysis, and cytokine release. This enables the T cell to redirect its specificity in an MHC-independent manner. See for example, Eshhar, Z., *Cancer Immunol Immunother*, 45(3-4):131-6, 1997 and Hwu, P., et al, *Cancer Res*, 55(15):3369-73, 1995. Another embodiment may include the transfection of tumor antigen specific alpha and beta T cell receptor chains into alternate T cells, as in Cole, DJ, et al, *Cancer Res*, 55(4):748-52, 1995.

In a further embodiment, syngeneic or autologous dendritic cells may be pulsed with peptides corresponding to at least an immunogenic portion of a polypeptide disclosed herein. The resulting antigen-specific dendritic cells may either be transferred into a patient, or employed to stimulate T cells to provide antigen-specific T cells which may, in turn, be administered to a patient. The use of peptide-pulsed dendritic cells to generate antigen-specific T cells and the subsequent use of such antigen-specific T cells to eradicate tumors in a murine model has been demonstrated by Cheever et al, *Immunological Reviews*, 157:177, 1997).

Additionally, vectors expressing the disclosed polynucleotides may be introduced into stem cells taken from the patient and clonally propagated *in vitro* for autologous transplant back into the same patient.

In general, an appropriate dosage and treatment regimen provides the active compound(s) in an amount sufficient to provide therapeutic and/or prophylactic benefit. Such a response can be monitored by establishing an improved clinical outcome (*e.g.*, more frequent remissions, complete or partial, or longer disease-free survival) in treated patients as compared to non-treated patients. Increases in preexisting immune responses to a prostate tumor protein generally correlate with an improved clinical outcome. Such immune responses may generally be evaluated using standard proliferation, cytotoxicity or cytokine assays, which may be performed using samples obtained from a patient before and after treatment.

METHODS FOR DETECTING CANCER

In general, a cancer may be detected in a patient based on the presence of one or more prostate tumor proteins and/or polynucleotides encoding such proteins in a biological sample obtained from the patient. In other words, such proteins may be used as markers to indicate the presence or absence of prostate cancer. The binding agents provided herein generally permit detection of the level of protein that binds to the agent in the biological sample. Alternatively, polynucleotide primers and probes may be used to detect the level of mRNA encoding an antigen, which is also indicative of the presence or absence of prostate cancer.

There are a variety of assay formats known to those of ordinary skill in the art for using a binding agent to detect polypeptide markers in a sample. *See, e.g.,* Harlow and Lane, *Antibodies: A Laboratory Manual*, Cold Spring Harbor Laboratory, 1988. In general, the presence or absence of a cancer in a patient may be determined by (a) contacting a biological sample obtained from a patient with a binding agent; (b) detecting in the sample a level of polypeptide that binds to the binding agent; and (c) comparing the level of polypeptide with a predetermined cut-off value.

In a preferred embodiment, the assay involves the use of binding agent immobilized on a solid support to bind to and remove the polypeptide from the remainder of the sample. The bound polypeptide may then be detected using a detection reagent that contains a reporter group and specifically binds to the binding agent/polypeptide complex. Such detection reagents may comprise, for example, a binding agent that specifically binds to the polypeptide or an antibody or other agent that specifically binds to the binding agent, such as an anti-immunoglobulin, protein G, protein A or a lectin. Alternatively, a competitive assay may be utilized, in which a polypeptide is labeled with a reporter group and allowed to bind to the immobilized binding agent after incubation of the binding agent with the sample. The extent to which components of the sample inhibit the binding of the labeled polypeptide to the binding agent is indicative of the reactivity of the sample with the immobilized binding agent. Suitable polypeptides for use within such assays include full length prostate

tumor proteins and portions thereof to which the binding agent binds, as described above.

The solid support may be any material known to those of ordinary skill in the art to which the antigen may be attached. For example, the solid support may be a test well in a microtiter plate or a nitrocellulose or other suitable membrane. Alternatively, the support may be a bead or disc, such as glass, fiberglass, latex or a plastic material such as polystyrene or polyvinylchloride. The support may also be a magnetic particle or a fiber optic sensor, such as those disclosed, for example, in U.S. Patent No. 5,359,681. The binding agent may be immobilized on the solid support using a variety of techniques known to those of skill in the art, which are amply described in the patent and scientific literature. In the context of the present invention, the term "immobilization" refers to both noncovalent association, such as adsorption, and covalent attachment (which may be a direct linkage between the antigen and functional groups on the support or may be a linkage by way of a cross-linking agent). Immobilization by adsorption to a well in a microtiter plate or to a membrane is preferred. In such cases, adsorption may be achieved by contacting the binding agent, in a suitable buffer, with the solid support for a suitable amount of time. The contact time varies with temperature, but is typically between about 1 hour and about 1 day. In general, contacting a well of a plastic microtiter plate (such as polystyrene or polyvinylchloride) with an amount of binding agent ranging from about 10 ng to about 10 μ g, and preferably about 100 ng to about 1 μ g, is sufficient to immobilize an adequate amount of binding agent.

Covalent attachment of binding agent to a solid support may generally be achieved by first reacting the support with a bifunctional reagent that will react with both the support and a functional group, such as a hydroxyl or amino group, on the binding agent. For example, the binding agent may be covalently attached to supports having an appropriate polymer coating using benzoquinone or by condensation of an aldehyde group on the support with an amine and an active hydrogen on the binding partner (*see, e.g.*, Pierce Immunotechnology Catalog and Handbook, 1991, at A12-A13).

In certain embodiments, the assay is a two-antibody sandwich assay. This assay may be performed by first contacting an antibody that has been immobilized on a solid support, commonly the well of a microtiter plate, with the sample, such that polypeptides within the sample are allowed to bind to the immobilized antibody.

5 Unbound sample is then removed from the immobilized polypeptide-antibody complexes and a detection reagent (preferably a second antibody capable of binding to a different site on the polypeptide) containing a reporter group is added. The amount of detection reagent that remains bound to the solid support is then determined using a method appropriate for the specific reporter group.

10 More specifically, once the antibody is immobilized on the support as described above, the remaining protein binding sites on the support are typically blocked. Any suitable blocking agent known to those of ordinary skill in the art, such as bovine serum albumin or Tween 20™ (Sigma Chemical Co., St. Louis, MO). The immobilized antibody is then incubated with the sample, and polypeptide is allowed to
15 bind to the antibody. The sample may be diluted with a suitable diluent, such as phosphate-buffered saline (PBS) prior to incubation. In general, an appropriate contact time (*i.e.*, incubation time) is a period of time that is sufficient to detect the presence of polypeptide within a sample obtained from an individual with prostate cancer. Preferably, the contact time is sufficient to achieve a level of binding that is at least
20 about 95% of that achieved at equilibrium between bound and unbound polypeptide. Those of ordinary skill in the art will recognize that the time necessary to achieve equilibrium may be readily determined by assaying the level of binding that occurs over a period of time. At room temperature, an incubation time of about 30 minutes is generally sufficient.

25 Unbound sample may then be removed by washing the solid support with an appropriate buffer, such as PBS containing 0.1% Tween 20™. The second antibody, which contains a reporter group, may then be added to the solid support. Preferred reporter groups include those groups recited above.

The detection reagent is then incubated with the immobilized antibody-
30 polypeptide complex for an amount of time sufficient to detect the bound polypeptide.

An appropriate amount of time may generally be determined by assaying the level of binding that occurs over a period of time. Unbound detection reagent is then removed and bound detection reagent is detected using the reporter group. The method employed for detecting the reporter group depends upon the nature of the reporter group. For radioactive groups, scintillation counting or autoradiographic methods are generally appropriate. Spectroscopic methods may be used to detect dyes, luminescent groups and fluorescent groups. Biotin may be detected using avidin, coupled to a different reporter group (commonly a radioactive or fluorescent group or an enzyme). Enzyme reporter groups may generally be detected by the addition of substrate (generally for a specific period of time), followed by spectroscopic or other analysis of the reaction products.

To determine the presence or absence of prostate cancer, the signal detected from the reporter group that remains bound to the solid support is generally compared to a signal that corresponds to a predetermined cut-off value. In one preferred embodiment, the cut-off value for the detection of prostate cancer is the average mean signal obtained when the immobilized antibody is incubated with samples from patients without prostate cancer. In general, a sample generating a signal that is three standard deviations above the predetermined cut-off value is considered positive for prostate cancer. In an alternate preferred embodiment, the cut-off value is determined using a Receiver Operator Curve, according to the method of Sackett et al., *Clinical Epidemiology: A Basic Science for Clinical Medicine*, Little Brown and Co., 1985, p. 106-7. Briefly, in this embodiment, the cut-off value may be determined from a plot of pairs of true positive rates (*i.e.*, sensitivity) and false positive rates (100%-specificity) that correspond to each possible cut-off value for the diagnostic test result. The cut-off value on the plot that is the closest to the upper left-hand corner (*i.e.*, the value that encloses the largest area) is the most accurate cut-off value, and a sample generating a signal that is higher than the cut-off value determined by this method may be considered positive. Alternatively, the cut-off value may be shifted to the left along the plot, to minimize the false positive rate, or to the right, to minimize the false negative rate. In general, a sample generating a signal that is higher than the cut-off

value determined by this method is considered positive for prostate cancer.

In a related embodiment, the assay is performed in a flow-through or strip test format, wherein the binding agent is immobilized on a membrane, such as nitrocellulose. In the flow-through test, polypeptides within the sample bind to the immobilized binding agent as the sample passes through the membrane. A second, labeled binding agent then binds to the binding agent-polypeptide complex as a solution containing the second binding agent flows through the membrane. The detection of bound second binding agent may then be performed as described above. In the strip test format, one end of the membrane to which binding agent is bound is immersed in a solution containing the sample. The sample migrates along the membrane through a region containing second binding agent and to the area of immobilized binding agent. Concentration of second binding agent at the area of immobilized antibody indicates the presence of prostate cancer. Typically, the concentration of second binding agent at that site generates a pattern, such as a line, that can be read visually. The absence of such a pattern indicates a negative result. In general, the amount of binding agent immobilized on the membrane is selected to generate a visually discernible pattern when the biological sample contains a level of polypeptide that would be sufficient to generate a positive signal in the two-antibody sandwich assay, in the format discussed above. Preferred binding agents for use in such assays are antibodies and antigen-binding fragments thereof. Preferably, the amount of antibody immobilized on the membrane ranges from about 25 ng to about 1 μ g, and more preferably from about 50 ng to about 500 ng. Such tests can typically be performed with a very small amount of biological sample.

Of course, numerous other assay protocols exist that are suitable for use with the antigens or binding agents of the present invention. The above descriptions are intended to be exemplary only.

In another embodiment, the above polypeptides may be used as markers for the progression of prostate cancer. In this embodiment, assays as described above for the diagnosis of prostate cancer may be performed over time, and the change in the level of reactive polypeptide(s) evaluated. For example, the assays may be performed

every 24-72 hours for a period of 6 months to 1 year, and thereafter performed as needed. In general, prostate cancer is progressing in those patients in whom the level of polypeptide detected by the binding agent increases over time. In contrast, the cancer is not progressing when the level of reactive polypeptide either remains constant or decreases with time.

As noted above, prostate cancer may also, or alternatively, be detected based on the level of mRNA encoding a prostate tumor protein in a biological sample. For example, at least two oligonucleotide primers may be employed in a polymerase chain reaction (PCR) based assay to amplify a portion of a prostate tumor protein cDNA derived from a biological sample, wherein at least one of the oligonucleotide primers is specific for (*i.e.*, hybridizes to) a polynucleotide encoding the prostate tumor protein. The amplified cDNA is then separated and detected using techniques well known in the art, such as gel electrophoresis. Similarly, oligonucleotide probes that specifically hybridize to a polynucleotide encoding a prostate tumor protein may be used in a hybridization assay to detect the presence of polynucleotide encoding the antigen in a biological sample.

To permit hybridization under assay conditions, oligonucleotide primers and probes should comprise an oligonucleotide sequence that has at least about 60%, preferably at least about 75% and more preferably at least about 90%, identity to a portion of a polynucleotide encoding a prostate tumor protein that is at least 10 nucleotides, and preferably at least 20 nucleotides, in length. Oligonucleotide primers and/or probes which may be usefully employed in the diagnostic methods described herein preferably are at least 10-40 nucleotides in length. In a preferred embodiment, the oligonucleotide primers comprise at least 10 contiguous nucleotides, more preferably at least 15 contiguous nucleotides, of a DNA molecule recited herein. Techniques for both PCR based assays and hybridization assays are well known in the art (*see*, for example, Mullis et al., *Cold Spring Harbor Symp. Quant. Biol.*, 51:263, 1987; Erlich ed., *PCR Technology*, Stockton Press, NY, 1989).

One preferred assay employs RT-PCR, in which PCR is applied in conjunction with reverse transcription. Typically, RNA is extracted from a sample

tissue and is reverse transcribed to produce cDNA molecules. PCR amplification using at least one specific primer generates a cDNA molecule, which may be separated and visualized using, for example, gel electrophoresis. Amplification may be performed on samples obtained from biological samples taken from a test patient and an individual
 5 who is not afflicted with prostate cancer. The amplification reaction may be performed on several dilutions of cDNA spanning two orders of magnitude. A two-fold or greater increase in expression in several dilutions of the test patient sample as compared to the same dilutions of the non-cancerous sample is typically considered positive.

Certain *in vivo* diagnostic assays may be performed directly on a tumor.
 10 One such assay involves contacting tumor cells with a binding agent. The bound binding agent may then be detected directly or indirectly via a reporter group. Such binding agents may also be used in histological applications. Alternatively, polynucleotide probes may be used within such applications.

As noted above, to improve sensitivity, multiple prostate tumor protein
 15 markers may be assayed within a given sample. It will be apparent that binding agents specific for different antigens provided herein may be combined within a single assay. Further, multiple primers or probes may be used concurrently. The selection of antigen markers may be based on routine experiments to determine combinations that results in optimal sensitivity. In addition, or alternatively, assays for antigens provided herein
 20 may be combined with assays for other known tumor antigens.

DIAGNOSTIC KITS

The present invention further provides kits for use within any of the above diagnostic methods. Such kits typically comprise two or more components
 25 necessary for performing a diagnostic assay. Components may be compounds, reagents, containers and/or equipment. For example, one container within a kit may contain a monoclonal antibody or fragment thereof that specifically binds to a prostate tumor protein. Such antibodies or fragments may be provided attached to a support material, as described above. One or more additional containers may enclose elements,
 30 such as reagents or buffers, to be used in the assay. Such kits may also, or alternatively,

contain a detection reagent as described above that contains a reporter group suitable for direct or indirect detection of antibody binding.

Alternatively, a kit may be designed to detect the level of mRNA encoding a prostate tumor protein in a biological sample. Such kits generally comprise at least one oligonucleotide probe or primer, as described above, that hybridizes to a polynucleotide encoding a prostate tumor protein. Such an oligonucleotide may be used, for example, within a PCR or hybridization assay. Additional components that may be present within such kits include a second oligonucleotide and/or a diagnostic reagent or container to facilitate the detection of a polynucleotide encoding a prostate tumor protein.

The following Examples are offered by way of illustration and not by way of limitation.

EXAMPLES

Example 1

ISOLATION AND CHARACTERIZATION OF PROSTATE TUMOR POLYPEPTIDES

This Example describes the isolation of certain prostate tumor polypeptides from a prostate tumor cDNA library.

A human prostate tumor cDNA expression library was constructed from prostate tumor poly A⁺ RNA using a Superscript Plasmid System for cDNA Synthesis and Plasmid Cloning kit (BRL Life Technologies, Gaithersburg, MD 20897) following the manufacturer's protocol. Specifically, prostate tumor tissues were homogenized with polytron (Kinematica, Switzerland) and total RNA was extracted using Trizol reagent (BRL Life Technologies) as directed by the manufacturer. The poly A⁺ RNA was then purified using a Qiagen oligotex spin column mRNA purification kit (Qiagen, Santa Clarita, CA 91355) according to the manufacturer's protocol. First-strand cDNA was synthesized using the NotI/Oligo-dT18 primer. Double-stranded cDNA was

synthesized, ligated with EcoRI/BAXI adaptors (Invitrogen, San Diego, CA) and digested with NotI. Following size fractionation with Chroma Spin-1000 columns (Clontech, Palo Alto, CA), the cDNA was ligated into the EcoRI/NotI site of pCDNA3.1 (Invitrogen) and transformed into ElectroMax *E. coli* DH10B cells (BRL Life Technologies) by electroporation.

Using the same procedure, a normal human pancreas cDNA expression library was prepared from a pool of six tissue specimens (Clontech). The cDNA libraries were characterized by determining the number of independent colonies, the percentage of clones that carried insert, the average insert size and by sequence analysis. The prostate tumor library contained 1.64×10^7 independent colonies, with 70% of clones having an insert and the average insert size being 1745 base pairs. The normal pancreas cDNA library contained 3.3×10^6 independent colonies, with 69% of clones having inserts and the average insert size being 1120 base pairs. For both libraries, sequence analysis showed that the majority of clones had a full length cDNA sequence and were synthesized from mRNA, with minimal rRNA and mitochondrial DNA contamination.

cDNA library subtraction was performed using the above prostate tumor and normal pancreas cDNA libraries, as described by Hara *et al.* (*Blood*, 84:189-199, 1994) with some modifications. Specifically, a prostate tumor-specific subtracted cDNA library was generated as follows. Normal pancreas cDNA library (70 μ g) was digested with EcoRI, NotI, and SfuI, followed by a filling-in reaction with DNA polymerase Klenow fragment. After phenol-chloroform extraction and ethanol precipitation, the DNA was dissolved in 100 μ l of H₂O, heat-denatured and mixed with 100 μ l (100 μ g) of Photoprobe biotin (Vector Laboratories, Burlingame, CA). As recommended by the manufacturer, the resulting mixture was irradiated with a 270 W sunlamp on ice for 20 minutes. Additional Photoprobe biotin (50 μ l) was added and the biotinylation reaction was repeated. After extraction with butanol five times, the DNA was ethanol-precipitated and dissolved in 23 μ l H₂O to form the driver DNA.

To form the tracer DNA, 10 μ g prostate tumor cDNA library was digested with BamHI and XhoI, phenol chloroform extracted and passed through

Chroma spin-400 columns (Clontech). Following ethanol precipitation, the tracer DNA was dissolved in 5 μ l H₂O. Tracer DNA was mixed with 15 μ l driver DNA and 20 μ l of 2 x hybridization buffer (1.5 M NaCl/10 mM EDTA/50 mM HEPES pH 7.5/0.2% sodium dodecyl sulfate), overlaid with mineral oil, and heat-denatured completely. The sample was immediately transferred into a 68 °C water bath and incubated for 20 hours (long hybridization [LH]). The reaction mixture was then subjected to a streptavidin treatment followed by phenol/chloroform extraction. This process was repeated three more times. Subtracted DNA was precipitated, dissolved in 12 μ l H₂O, mixed with 8 μ l driver DNA and 20 μ l of 2 x hybridization buffer, and subjected to a hybridization at 68 °C for 2 hours (short hybridization [SH]). After removal of biotinylated double-stranded DNA, subtracted cDNA was ligated into BamHI/XhoI site of chloramphenicol resistant pBCSK⁺ (Stratagene, La Jolla, CA 92037) and transformed into ElectroMax *E. coli* DH10B cells by electroporation to generate a prostate tumor specific subtracted cDNA library (prostate subtraction 1).

To analyze the subtracted cDNA library, plasmid DNA was prepared from 100 independent clones, randomly picked from the subtracted prostate tumor specific library and grouped based on insert size. Representative cDNA clones were further characterized by DNA sequencing with a Perkin Elmer/Applied Biosystems Division Automated Sequencer Model 373A (Foster City, CA). Six cDNA clones, hereinafter referred to as F1-13, F1-12, F1-16, H1-1, H1-9 and H1-4, were shown to be abundant in the subtracted prostate-specific cDNA library. The determined 3' and 5' cDNA sequences for F1-12 are provided in SEQ ID NO: 2 and 3, respectively, with determined 3' cDNA sequences for F1-13, F1-16, H1-1, H1-9 and H1-4 being provided in SEQ ID NO: 1 and 4-7, respectively.

The cDNA sequences for the isolated clones were compared to known sequences in the gene bank using the EMBL and GenBank databases (release 96). Four of the prostate tumor cDNA clones, F1-13, F1-16, H1-1, and H1-4, were determined to encode the following previously identified proteins: prostate specific antigen (PSA), human glandular kallikrein, human tumor expression enhanced gene, and mitochondria cytochrome C oxidase subunit II. H1-9 was found to be identical to a previously

identified human autonomously replicating sequence. No significant homologies to the cDNA sequence for F1-12 were found.

Subsequent studies led to the isolation of a full-length cDNA sequence for F1-12. This sequence is provided in SEQ ID NO: 107, with the corresponding
 5 predicted amino acid sequence being provided in SEQ ID NO: 108.

To clone less abundant prostate tumor specific genes, cDNA library subtraction was performed by subtracting the prostate tumor cDNA library described above with the normal pancreas cDNA library and with the three most abundant genes in the previously subtracted prostate tumor specific cDNA library: human glandular
 10 kallikrein, prostate specific antigen (PSA), and mitochondria cytochrome C oxidase subunit II. Specifically, 1 μ g each of human glandular kallikrein, PSA and mitochondria cytochrome C oxidase subunit II cDNAs in pCDNA3.1 were added to the driver DNA and subtraction was performed as described above to provide a second subtracted cDNA library hereinafter referred to as the "subtracted prostate tumor
 15 specific cDNA library with spike".

Twenty-two cDNA clones were isolated from the subtracted prostate tumor specific cDNA library with spike. The determined 3' and 5' cDNA sequences for the clones referred to as J1-17, L1-12, N1-1862, J1-13, J1-19, J1-25, J1-24, K1-58, K1-63, L1-4 and L1-14 are provided in SEQ ID NOS: 8-9, 10-11, 12-13, 14-15, 16-17, 18-
 20 19, 20-21, 22-23, 24-25, 26-27 and 28-29, respectively. The determined 3' cDNA sequences for the clones referred to as J1-12, J1-16, J1-21, K1-48, K1-55, L1-2, L1-6, N1-1858, N1-1860, N1-1861, N1-1864 are provided in SEQ ID NOS: 30-40, respectively. Comparison of these sequences with those in the gene bank as described above, revealed no significant homologies to three of the five most abundant DNA
 25 species, (J1-17, L1-12 and N1-1862; SEQ ID NOS: 8-9, 10-11 and 12-13, respectively). Of the remaining two most abundant species, one (J1-12; SEQ ID NO:30) was found to be identical to the previously identified human pulmonary surfactant-associated protein, and the other (K1-48; SEQ ID NO:33) was determined to have some homology to *R. norvegicus* mRNA for 2-arylpropionyl-CoA epimerase. Of the 17 less abundant cDNA
 30 clones isolated from the subtracted prostate tumor specific cDNA library with spike,

four (J1-16, K1-55, L1-6 and N1-1864; SEQ ID NOS:31, 34, 36 and 40, respectively) were found to be identical to previously identified sequences, two (J1-21 and N1-1860; SEQ ID NOS: 32 and 38, respectively) were found to show some homology to non-human sequences, and two (L1-2 and N1-1861; SEQ ID NOS: 35 and 39, respectively) were found to show some homology to known human sequences. No significant homologies were found to the polypeptides J1-13, J1-19, J1-24, J1-25, K1-58, K1-63, L1-4, L1-14 (SEQ ID NOS: 14-15, 16-17, 20-21, 18-19, 22-23, 24-25, 26-27, 28-29, respectively).

Subsequent studies led to the isolation of full length cDNA sequences for J1-17, L1-12 and N1-1862 (SEQ ID NOS: 109-111, respectively). The corresponding predicted amino acid sequences are provided in SEQ ID NOS: 112-114. L1-12 is also referred to as P501S.

In a further experiment, four additional clones were identified by subtracting a prostate tumor cDNA library with normal prostate cDNA prepared from a pool of three normal prostate poly A+ RNA (prostate subtraction 2). The determined cDNA sequences for these clones, hereinafter referred to as U1-3064, U1-3065, V1-3692 and 1A-3905, are provided in SEQ ID NO: 69-72, respectively. Comparison of the determined sequences with those in the gene bank revealed no significant homologies to U1-3065.

A second subtraction with spike (prostate subtraction spike 2) was performed by subtracting a prostate tumor specific cDNA library with spike with normal pancreas cDNA library and further spiked with PSA, J1-17, pulmonary surfactant-associated protein, mitochondrial DNA, cytochrome c oxidase subunit II, N1-1862, autonomously replicating sequence, L1-12 and tumor expression enhanced gene. Four additional clones, hereinafter referred to as V1-3686, R1-2330, 1B-3976 and V1-3679, were isolated. The determined cDNA sequences for these clones are provided in SEQ ID NO:73-76, respectively. Comparison of these sequences with those in the gene bank revealed no significant homologies to V1-3686 and R1-2330.

Further analysis of the three prostate subtractions described above (prostate subtraction 2, subtracted prostate tumor specific cDNA library with spike, and

prostate subtraction spike 2) resulted in the identification of sixteen additional clones, referred to as 1G-4736, 1G-4738, 1G-4741, 1G-4744, 1G-4734, 1H-4774, 1H-4781, 1H-4785, 1H-4787, 1H-4796, 1I-4810, 1I-4811, 1J-4876, 1K-4884 and 1K-4896. The determined cDNA sequences for these clones are provided in SEQ ID NOS: 77-92, respectively. Comparison of these sequences with those in the gene bank as described above, revealed no significant homologies to 1G-4741, 1G-4734, 1I-4807, 1J-4876 and 1K-4896 (SEQ ID NOS: 79, 81, 87, 90 and 92, respectively). Further analysis of the isolated clones led to the determination of extended cDNA sequences for 1G-4736, 1G-4738, 1G-4741, 1G-4744, 1H-4774, 1H-4781, 1H-4785, 1H-4787, 1H-4796, 1I-4807, 1J-4876, 1K-4884 and 1K-4896, provided in SEQ ID NOS: 179-188 and 191-193, respectively, and to the determination of additional partial cDNA sequences for 1I-4810 and 1I-4811, provided in SEQ ID NOS: 189 and 190, respectively.

Additional studies with prostate subtraction spike 2 resulted in the isolation of three more clones. Their sequences were determined as described above and compared to the most recent Genbank. All three clones were found to have homology to known genes, which are Cysteine-rich protein, KIAA0242, and KIAA0280 (SEQ ID NO: 317, 319, and 320, respectively). Further analysis of these clones by Synteni microarray (Synteni, Palo Alto, CA) demonstrated that all three clones were over-expressed in most prostate tumors and prostate BPH, as well as in the majority of normal prostate tissues tested, but low expression in all other normal tissues.

An additional subtraction was performed by subtracting a normal prostate cDNA library with normal pancreas cDNA (prostate subtraction 3). This led to the identification of six additional clones referred to as 1G-4761, 1G-4762, 1H-4766, 1H-4770, 1H-4771 and 1H-4772 (SEQ ID NOS: 93-98). Comparison of these sequences with those in the gene bank revealed no significant homologies to 1G-4761 and 1H-4771 (SEQ ID NOS: 93 and 97, respectively). Further analysis of the isolated clones led to the determination of extended cDNA sequences for 1G-4761, 1G-4762, 1H-4766 and 1H-4772 provided in SEQ ID NOS: 194-196 and 199, respectively, and to the determination of additional partial cDNA sequences for 1H-4770 and 1H-4771, provided in SEQ ID NOS: 197 and 198, respectively.

Subtraction of a prostate tumor cDNA library, prepared from a pool of polyA⁺ RNA from three prostate cancer patients, with a normal pancreas cDNA library (prostate subtraction 4) led to the identification of eight clones, referred to as 1D-4297, 1D-4309, 1D-4278, 1D-4288, 1D-4283, 1D-4304, 1D-4296 and 1D-4280 (SEQ ID NOS: 99-107). These sequences were compared to those in the gene bank as described above. No significant homologies were found to 1D-4283 and 1D-4304 (SEQ ID NOS: 103 and 104, respectively). Further analysis of the isolated clones led to the determination of extended cDNA sequences for 1D-4309, 1D-4278, 1D-4288, 1D-4283, 1D-4304, 1D-4296 and 1D-4280, provided in SEQ ID NOS: 200-206, respectively.

cDNA clones isolated in prostate subtraction 1 and prostate subtraction 2, described above, were colony PCR amplified and their mRNA expression levels in prostate tumor, normal prostate and in various other normal tissues were determined using microarray technology (Synteni, Palo Alto, CA). Briefly, the PCR amplification products were dotted onto slides in an array format, with each product occupying a unique location in the array. mRNA was extracted from the tissue sample to be tested, reverse transcribed, and fluorescent-labeled cDNA probes were generated. The microarrays were probed with the labeled cDNA probes, the slides scanned and fluorescence intensity was measured. This intensity correlates with the hybridization intensity. Two novel clones (referred to as P509S and P510S) were found to be over-expressed in prostate tumor and normal prostate and expressed at low levels in all other normal tissues tested (liver, pancreas, skin, bone marrow, brain, breast, adrenal gland, bladder, testes, salivary gland, large intestine, kidney, ovary, lung, spinal cord, skeletal muscle and colon). The determined cDNA sequences for P509S and P510S are provided in SEQ ID NO: 223 and 224, respectively. Comparison of these sequences with those in the gene bank as described above, revealed some homology to previously identified ESTs.

Additionally, the full-length cDNA sequence for P509S (SEQ ID NO: 223) is provided in SEQ ID NO: 332.

Example 2

DETERMINATION OF TISSUE SPECIFICITY OF PROSTATE TUMOR
POLYPEPTIDES

5 Using gene specific primers, mRNA expression levels for the representative prostate tumor polypeptides F1-16, H1-1, J1-17 (also referred to as P502S), L1-12 (also referred to as P501S), F1-12 (also referred to as P504S) and N1-1862 (also referred to as P503S) were examined in a variety of normal and tumor tissues using RT-PCR.

10 Briefly, total RNA was extracted from a variety of normal and tumor tissues using Trizol reagent as described above. First strand synthesis was carried out using 1-2 μ g of total RNA with SuperScript II reverse transcriptase (BRL Life Technologies) at 42 °C for one hour. The cDNA was then amplified by PCR with gene-specific primers. To ensure the semi-quantitative nature of the RT-PCR, β -actin was
15 used as an internal control for each of the tissues examined. First, serial dilutions of the first strand cDNAs were prepared and RT-PCR assays were performed using β -actin specific primers. A dilution was then chosen that enabled the linear range amplification of the β -actin template and which was sensitive enough to reflect the differences in the initial copy numbers. Using these conditions, the β -actin levels were determined for
20 each reverse transcription reaction from each tissue. DNA contamination was minimized by DNase treatment and by assuring a negative PCR result when using first strand cDNA that was prepared without adding reverse transcriptase.

 mRNA Expression levels were examined in four different types of tumor tissue (prostate tumor from 2 patients, breast tumor from 3 patients, colon tumor, lung
25 tumor), and sixteen different normal tissues, including prostate, colon, kidney, liver, lung, ovary, pancreas, skeletal muscle, skin, stomach, testes, bone marrow and brain. F1-16 was found to be expressed at high levels in prostate tumor tissue, colon tumor and normal prostate, and at lower levels in normal liver, skin and testes, with expression being undetectable in the other tissues examined. H1-1 was found to be expressed at
30 high levels in prostate tumor, lung tumor, breast tumor, normal prostate, normal colon

and normal brain, at much lower levels in normal lung, pancreas, skeletal muscle, skin, small intestine, bone marrow, and was not detected in the other tissues tested. J1-17 (P502S) and L1-12 (P501S) appear to be specifically over-expressed in prostate, with both genes being expressed at high levels in prostate tumor and normal prostate but at low to undetectable levels in all the other tissues examined. N1-1862 (P503S) was found to be over-expressed in 60% of prostate tumors and detectable in normal colon and kidney. The RT-PCR results thus indicate that F1-16, H1-1, J1-17 (P502S), N1-1862 (P503S) and L1-12 (P501S) are either prostate specific or are expressed at significantly elevated levels in prostate.

Further RT-PCR studies showed that F1-12 (P504S) is over-expressed in 60% of prostate tumors, detectable in normal kidney but not detectable in all other tissues tested. Similarly, R1-2330 was shown to be over-expressed in 40% of prostate tumors, detectable in normal kidney and liver, but not detectable in all other tissues tested. U1-3064 was found to be over-expressed in 60% of prostate tumors, and also expressed in breast and colon tumors, but was not detectable in normal tissues.

RT-PCR characterization of R1-2330, U1-3064 and 1D-4279 showed that these three antigens are over-expressed in prostate and/or prostate tumors.

Northern analysis with four prostate tumors, two normal prostate samples, two BPH prostates, and normal colon, kidney, liver, lung, pancreas, skeletal muscle, brain, stomach, testes, small intestine and bone marrow, showed that L1-12 (P501S) is over-expressed in prostate tumors and normal prostate, while being undetectable in other normal tissues tested. J1-17 (P502S) was detected in two prostate tumors and not in the other tissues tested. N1-1862 (P503S) was found to be over-expressed in three prostate tumors and to be expressed in normal prostate, colon and kidney, but not in other tissues tested. F1-12 (P504S) was found to be highly expressed in two prostate tumors and to be undetectable in all other tissues tested.

The micro-array technology described above was used to determine the expression levels of representative antigens described herein in prostate tumor, breast tumor and the following normal tissues: prostate, liver, pancreas, skin, bone marrow, brain, breast, adrenal gland, bladder, testes, salivary gland, large intestine, kidney,

ovary, lung, spinal cord, skeletal muscle and colon. L1-12 (P501S) was found to be over-expressed in normal prostate and prostate tumor, with some expression being detected in normal skeletal muscle. Both J1-12 and F1-12 (P504S) were found to be over-expressed in prostate tumor, with expression being lower or undetectable in all other tissues tested. N1-1862 (P503S) was found to be expressed at high levels in prostate tumor and normal prostate, and at low levels in normal large intestine and normal colon, with expression being undetectable in all other tissues tested. R1-2330 was found to be over-expressed in prostate tumor and normal prostate, and to be expressed at lower levels in all other tissues tested. 1D-4279 was found to be over-expressed in prostate tumor and normal prostate, expressed at lower levels in normal spinal cord, and to be undetectable in all other tissues tested.

Further studies to specifically address the extent to which P501S (SEQ ID NO: 110) was expressed in breast tumor by microarray analysis revealed moderate over-expression in not only breast tumor, but also in metastatic breast tumor (2/31), with negligible to low expression in normal tissues. This data demonstrates that P501S may be over-expressed in various breast tumors as well as in prostate tumors.

Example 3

ISOLATION AND CHARACTERIZATION OF PROSTATE TUMOR POLYPEPTIDES BY PCR-BASED SUBTRACTION

A cDNA subtraction library, containing cDNA from normal prostate subtracted with ten other normal tissue cDNAs (brain, heart, kidney, liver, lung, ovary, placenta, skeletal muscle, spleen and thymus) and then submitted to a first round of PCR amplification, was purchased from Clontech. This library was subjected to a second round of PCR amplification, following the manufacturer's protocol. The resulting cDNA fragments were subcloned into the vector pT7 Blue T-vector (Novagen, Madison, WI) and transformed into XL-1 Blue MRF' *E. coli* (Stratagene). DNA was isolated from independent clones and sequenced using a Perkin Elmer/Applied Biosystems Division Automated Sequencer Model 373A.

Fifty-nine positive clones were sequenced. Comparison of the DNA sequences of these clones with those in the gene bank, as described above, revealed no significant homologies to 25 of these clones, hereinafter referred to as P5, P8, P9, P18, P20, P30, P34, P36, P38, P39, P42, P49, P50, P53, P55, P60, P64, P65, P73, P75, P76, P79 and P84. The determined cDNA sequences for these clones are provided in SEQ ID NO:41-45, 47-52 and 54-65, respectively. P29, P47, P68, P80 and P82 (SEQ ID NO:46, 53 and 66-68, respectively) were found to show some degree of homology to previously identified DNA sequences. To the best of the inventors' knowledge, none of these sequences have been previously shown to be present in prostate.

Further studies using the PCR-based methodology described above resulted in the isolation of more than 180 additional clones, of which 23 clones were found to show no significant homologies to known sequences. The determined cDNA sequences for these clones are provided in SEQ ID NO: 115-123, 127, 131, 137, 145, 147-151, 153, 156-158 and 160. Twenty-three clones (SEQ ID NO: 124-126, 128-130, 132-136, 138-144, 146, 152, 154, 155 and 159) were found to show some homology to previously identified ESTs. An additional ten clones (SEQ ID NO: 161-170) were found to have some degree of homology to known genes. Larger cDNA clones containing the P20 sequence represent splice variants of a gene referred to as P703P. The determined DNA sequence for the variants referred to as DE1, DE13 and DE14 are provided in SEQ ID NOS: 171, 175 and 177, respectively, with the corresponding predicted amino acid sequences being provided in SEQ ID NO: 172, 176 and 178, respectively. The determined cDNA sequence for an extended spliced form of P703 is provided in SEQ ID NO: 225. The DNA sequences for the splice variants referred to as DE2 and DE6 are provided in SEQ ID NOS: 173 and 174, respectively.

mRNA Expression levels for representative clones in tumor tissues (prostate (n=5), breast (n=2), colon and lung) normal tissues (prostate (n=5), colon, kidney, liver, lung (n=2), ovary (n=2), skeletal muscle, skin, stomach, small intestine and brain), and activated and non-activated PBMC was determined by RT-PCR as described above. Expression was examined in one sample of each tissue type unless otherwise indicated.

P9 was found to be highly expressed in normal prostate and prostate tumor compared to all normal tissues tested except for normal colon which showed comparable expression. P20, a portion of the P703P gene, was found to be highly expressed in normal prostate and prostate tumor, compared to all twelve normal tissues tested. A modest increase in expression of P20 in breast tumor (n=2), colon tumor and lung tumor was seen compared to all normal tissues except lung (1 of 2). Increased expression of P18 was found in normal prostate, prostate tumor and breast tumor compared to other normal tissues except lung and stomach. A modest increase in expression of P5 was observed in normal prostate compared to most other normal tissues. However, some elevated expression was seen in normal lung and PBMC. Elevated expression of P5 was also observed in prostate tumors (2 of 5), breast tumor and one lung tumor sample. For P30, similar expression levels were seen in normal prostate and prostate tumor, compared to six of twelve other normal tissues tested. Increased expression was seen in breast tumors, one lung tumor sample and one colon tumor sample, and also in normal PBMC. P29 was found to be over-expressed in prostate tumor (5 of 5) and normal prostate (5 of 5) compared to the majority of normal tissues. However, substantial expression of P29 was observed in normal colon and normal lung (2 of 2). P80 was found to be over-expressed in prostate tumor (5 of 5) and normal prostate (5 of 5) compared to all other normal tissues tested, with increased expression also being seen in colon tumor.

Further studies resulted in the isolation of twelve additional clones, hereinafter referred to as 10-d8, 10-h10, 11-c8, 7-g6, 8-b5, 8-b6, 8-d4, 8-d9, 8-g3, 8-h11, 9-f12 and 9-f3. The determined DNA sequences for 10-d8, 10-h10, 11-c8, 8-d4, 8-d9, 8-h11, 9-f12 and 9-f3 are provided in SEQ ID NO: 207, 208, 209, 216, 217, 220, 221 and 222, respectively. The determined forward and reverse DNA sequences for 7-g6, 8-b5, 8-b6 and 8-g3 are provided in SEQ ID NO: 210 and 211; 212 and 213; 214 and 215; and 218 and 219, respectively. Comparison of these sequences with those in the gene bank revealed no significant homologies to the sequence of 9-f3. The clones 10-d8, 11-c8 and 8-h11 were found to show some homology to previously isolated ESTs, while 10-h10, 8-b5, 8-b6, 8-d4, 8-d9, 8-g3 and 9-f12 were found to show some

homology to previously identified genes. Further characterization of 7-G6 and 8-G3 showed identity to the known genes PAP and PSA, respectively.

mRNA expression levels for these clones were determined using the micro-array technology described above. The clones 7-G6, 8-G3, 8-B5, 8-B6, 8-D4, 8-D9, 9-F3, 9-F12, 9-H3, 10-A2, 10-A4, 11-C9 and 11-F2 were found to be over-expressed in prostate tumor and normal prostate, with expression in other tissues tested being low or undetectable. Increased expression of 8-F11 was seen in prostate tumor and normal prostate, bladder, skeletal muscle and colon. Increased expression of 10-H10 was seen in prostate tumor and normal prostate, bladder, lung, colon, brain and large intestine. Increased expression of 9-B1 was seen in prostate tumor, breast tumor, and normal prostate, salivary gland, large intestine and skin, with increased expression of 11-C8 being seen in prostate tumor, and normal prostate and large intestine.

An additional cDNA fragment derived from the PCR-based normal prostate subtraction, described above, was found to be prostate specific by both micro-array technology and RT-PCR. The determined cDNA sequence of this clone (referred to as 9-A11) is provided in SEQ ID NO: 226. Comparison of this sequence with those in the public databases revealed 99% identity to the known gene HOXB13.

Further studies led to the isolation of the clones 8-C6 and 8-H7. The determined cDNA sequences for these clones are provided in SEQ ID NO: 227 and 228, respectively. These sequences were found to show some homology to previously isolated ESTs.

PCR and hybridization-based methodologies were employed to obtain longer cDNA sequences for clone P20 (also referred to as P703P), yielding three additional cDNA fragments that progressively extend the 5' end of the gene. These fragments, referred to as P703PDE5, P703P6.26, and P703PX_23 (SEQ ID NO: 326, 328, and 330, with the predicted corresponding amino acid sequences in SEQ ID NO: 327, 329, and 331, respectively) contain additional 5' sequence. P703PDE5 was recovered by screening of a cDNA library (#141-26) with a portion of P703P as a probe. P703P6.26 was recovered from a mixture of three prostate tumor cDNAs and P703PX_23 was recovered from cDNA library (#438-48). Together, the additional

sequences include all of the putative mature serine protease along with the majority of the putative signal sequence. Further studies using a PCR-based subtraction library of a prostate tumor pool subtracted against a pool of normal tissues (referred to as JP: PCR subtraction) resulted in the isolation of thirteen additional clones, seven of which did not share any significant homology to known Genbank sequences. The determined cDNA sequences for novel clones P711P, P712P, P774P, P775P, novel 27, P710P, and P768P are provided in SEQ ID NO: 307-311, 313, and 315, respectively. The remaining six clones (SEQ ID NO: 316, and 321-325) were shown to share homology to known genes. By microarray analysis, all thirteen clones showed three or more fold over-expression in prostate tissues, including prostate tumors, BPH, and normal prostate as compared to normal non-prostate tissues. Clones P711P, P712P, novel 23, and P768P showed over-expression in most prostate tumors and BPH tissues tested (n=29), and in the majority of normal prostate tissues (n=4), but background to low expression levels in all normal tissues. Clones P774P, P775P, and P710P showed comparatively lower expression and expression in fewer prostate tumors and BPH samples, with negative to low expression in normal prostate.

Using PCR and hybridization-based methodologies, additional cDNA sequence information was derived for two clones described above, 11-C9 and 9-F3, herein after referred to as P707P and P714P, respectively (SEQ ID NO: 333 and 334). After comparison with the most recent Genbank, P707P was found to be a splice variant of the known gene HoxB13. While there are some differences in the published sequence and the derived cDNA sequence, the differences are likely due to allelic variation. In contrast, P714P does not share homology with any known gene sequence and therefore is novel.

Additionally, clones 8-B3, P89, P98, P130, and P201 (as disclosed in U.S. Patent Application No. 09/020,956, filed February 9, 1998) were found to be contained within one contiguous sequence, referred to as P705P (SEQ ID NO: 335, with the predicted amino acid sequence provided in SEQ ID NO: 336), which was determined to be a splice variant of the known gene NKX 3.1.

Example 4

SYNTHESIS OF POLYPEPTIDES

Polypeptides may be synthesized on a Perkin Elmer/Applied Biosystems
 5 430A peptide synthesizer using Fmoc chemistry with HPTU (O-Benzotriazole-
 N,N,N',N'-tetramethyluronium hexafluorophosphate) activation. A Gly-Cys-Gly
 sequence may be attached to the amino terminus of the peptide to provide a method of
 conjugation, binding to an immobilized surface, or labeling of the peptide. Cleavage of
 the peptides from the solid support may be carried out using the following cleavage
 10 mixture: trifluoroacetic acid:ethanedithiol:thioanisole:water:phenol (40:1:2:2:3). After
 cleaving for 2 hours, the peptides may be precipitated in cold methyl-t-butyl-ether. The
 peptide pellets may then be dissolved in water containing 0.1% trifluoroacetic acid
 (TFA) and lyophilized prior to purification by C18 reverse phase HPLC. A gradient of
 0%-60% acetonitrile (containing 0.1% TFA) in water (containing 0.1% TFA) may be
 15 used to elute the peptides. Following lyophilization of the pure fractions, the peptides
 may be characterized using electrospray or other types of mass spectrometry and by
 amino acid analysis.

Example 5

FURTHER ISOLATION AND CHARACTERIZATION OF PROSTATE TUMOR
 POLYPEPTIDES BY PCR-BASED SUBTRACTION

A cDNA library generated from prostate primary tumor mRNA as
 25 described above was subtracted with cDNA from normal prostate. The subtraction was
 performed using a PCR-based protocol (Clontech), which was modified to generate
 larger fragments. Within this protocol, tester and driver double stranded cDNA were
 separately digested with five restriction enzymes that recognize six-nucleotide
 restriction sites (MluI, MscI, PvuII, SalI and StuI). This digestion resulted in an
 30 average cDNA size of 600 bp, rather than the average size of 300 bp that results from

digestion with *RsaI* according to the Clontech protocol. This modification did not affect the subtraction efficiency. Two tester populations were then created with different adapters, and the driver library remained without adapters.

The tester and driver libraries were then hybridized using excess driver cDNA. In the first hybridization step, driver was separately hybridized with each of the two tester cDNA populations. This resulted in populations of (a) unhybridized tester cDNAs, (b) tester cDNAs hybridized to other tester cDNAs, (c) tester cDNAs hybridized to driver cDNAs and (d) unhybridized driver cDNAs. The two separate hybridization reactions were then combined, and rehybridized in the presence of additional denatured driver cDNA. Following this second hybridization, in addition to populations (a) through (d), a fifth population (e) was generated in which tester cDNA with one adapter hybridized to tester cDNA with the second adapter. Accordingly, the second hybridization step resulted in enrichment of differentially expressed sequences which could be used as templates for PCR amplification with adaptor-specific primers.

The ends were then filled in, and PCR amplification was performed using adaptor-specific primers. Only population (e), which contained tester cDNA that did not hybridize to driver cDNA, was amplified exponentially. A second PCR amplification step was then performed, to reduce background and further enrich differentially expressed sequences.

This PCR-based subtraction technique normalizes differentially expressed cDNAs so that rare transcripts that are overexpressed in prostate tumor tissue may be recoverable. Such transcripts would be difficult to recover by traditional subtraction methods.

In addition to genes known to be overexpressed in prostate tumor, seventy-seven novel clones were identified. Sequences of these partial cDNAs are provided in SEQ ID NOs:229 to 305. Most of these clones had no significant homology to database sequences. Exceptions were JPTPN23 (SEQ ID NO:231; similarity to pig valosin-containing protein), JPTPN30 (SEQ ID NO:234; similarity to rat mRNA for proteasome subunit), JPTPN45 (SEQ ID NO:243; similarity to rat *norvegicus* cytosolic NADP-dependent isocitrate dehydrogenase), JPTPN46 (SEQ ID

NO:244; similarity to human subclone H8 4 d4 DNA sequence), JP1D6 (SEQ ID NO:265; similarity to *G. gallus* dynein light chain-A), JP8D6 (SEQ ID NO:288; similarity to human BAC clone RG016J04), JP8F5 (SEQ ID NO:289; similarity to human subclone H8 3 b5 DNA sequence) and JP8E9 (SEQ ID NO:299; similarity to human Alu sequence).

The novel clones identified were:

	JTPN13	SEQ ID NO:229
	JTPN14	SEQ ID NO:230
10	JTPN23	SEQ ID NO:231
	JTPN24	SEQ ID NO:232
	JTPN25	SEQ ID NO:233
	JTPN30	SEQ ID NO:234
	JTPN34	SEQ ID NO:235
15	JTPN35	SEQ ID NO:236
	JTPN36	SEQ ID NO:237
	JTPN38	SEQ ID NO:238
	JTPN39	SEQ ID NO:239
	JTPN40	SEQ ID NO:240
20	JTPN41	SEQ ID NO:241
	JTPN42	SEQ ID NO:242
	JTPN45	SEQ ID NO:243
	JTPN46	SEQ ID NO:244
	JTPN51	SEQ ID NO:245
25	JTPN56	SEQ ID NO:246
	JTPN64	SEQ ID NO:247
	JTPN65	SEQ ID NO:248
	JTPN67	SEQ ID NO:249
	JTPN76	SEQ ID NO:250
30	JTPN84	SEQ ID NO:251

	JPTPN85	SEQ ID NO:252
	JPTPN86	SEQ ID NO:253
	JPTPN87	SEQ ID NO:254
	JPTPN88	SEQ ID NO:255
5	JP1F1	SEQ ID NO:256
	JP1F2	SEQ ID NO:257
	JP1C2	SEQ ID NO:258
	JP1B1	SEQ ID NO:259
	JP1B2	SEQ ID NO:260
10	JP1D3	SEQ ID NO:261
	JP1A4	SEQ ID NO:262
	JP1F5	SEQ ID NO:263
	JP1E6	SEQ ID NO:264
	JP1D6	SEQ ID NO:265
15	JP1B5	SEQ ID NO:266
	JP1A6	SEQ ID NO:267
	JP1E8	SEQ ID NO:268
	JP1D7	SEQ ID NO:269
	JP1D9	SEQ ID NO:270
20	JP1C10	SEQ ID NO:271
	JP1A9	SEQ ID NO:272
	JP1F12	SEQ ID NO:273
	JP1E12	SEQ ID NO:274
	JP1D11	SEQ ID NO:275
25	JP1C11	SEQ ID NO:276
	JP1C12	SEQ ID NO:277
	JP1B12	SEQ ID NO:278
	JP1A12	SEQ ID NO:279
	JP8G2	SEQ ID NO:280
30	JP8H1	SEQ ID NO:281

	JP8H2	SEQ ID NO:282
	JP8A3	SEQ ID NO:283
	JP8A4	SEQ ID NO:284
	JP8C3	SEQ ID NO:285
5	JP8G4	SEQ ID NO:286
	JP8B6	SEQ ID NO:287
	JP8D6	SEQ ID NO:288
	JP8F5	SEQ ID NO:289
	JP8A8	SEQ ID NO:290
10	JP8C7	SEQ ID NO:291
	JP8D7	SEQ ID NO:292
	JP8D8	SEQ ID NO:293
	JP8E7	SEQ ID NO:294
	JP8F8	SEQ ID NO:295
15	JP8G8	SEQ ID NO:296
	JP8B10	SEQ ID NO:297
	JP8C10	SEQ ID NO:298
	JP8E9	SEQ ID NO:299
	JP8E10	SEQ ID NO:300
20	JP8F9	SEQ ID NO:301
	JP8H9	SEQ ID NO:302
	JP8C12	SEQ ID NO:303
	JP8E11	SEQ ID NO:304
	JP8E12	SEQ ID NO:305
25		

Additional studies using the PCR-based subtraction library consisting of a prostate tumor pool subtracted against a normal prostate pool (referred to as PT-PN PCR subtraction) yielded three additional clones. Comparison of the cDNA sequences of these clones with the most recent Genbank revealed two to be novel, herein after referred to as P715P and P767P (SEQ ID NO: 312 and 314). The remaining clone was

shown to share homology to the known gene KIAA0056 (SEQ ID NO: 318). Using microarray analysis to measure mRNA expression levels in various tissues, all three clones were found to be over-expressed in prostate tumors and BPH tissues. Specifically, clone P715P was over-expressed in most prostate tumors and BPH tissues by a factor of three or greater, with elevated expression seen in the majority of normal prostate samples and in fetal tissue, but negative to low expression in all other normal tissues. Clone P767P was over-expressed in several prostate tumors and BPH tissues, with moderate expression levels in half of the normal prostate samples, and background to low expression in all other normal tissues tested.

Example 6

PEPTIDE PRIMING OF MICE AND PROPAGATION OF CTL LINES

6.1. This Example illustrates the preparation of a CTL cell line specific for cells expressing the P502S gene.

Mice expressing the transgene for human HLA A2.1 (provided by Dr L. Sherman, The Scripps Research Institute, La Jolla, CA) were immunized with P2S #12 peptide (VLGWVAEL; SEQ ID NO: 306), which is derived from the P502S gene (also referred to herein as J1-17, SEQ ID NO:8), as described by Theobald et al., *Proc. Natl. Acad. Sci. USA* 92:11993-11997, 1995 with the following modifications. Mice were immunized with 100µg of P2S #12 and 120µg of an I-A^b binding peptide derived from hepatitis B Virus protein emulsified in incomplete Freund's adjuvant. Three weeks later these mice were sacrificed and using a nylon mesh single cell suspensions prepared. Cells were then resuspended at 6×10^6 cells/ml in complete media (RPMI- 1640 (Gibco BRL, Gaithersburg, MD) containing 10% FCS, 2mM Glutamine (Gibco BRL), sodium pyruvate (Gibco BRL), non- essential amino acids (Gibco BRL, 2×10^{-5} M 2-mercaptoethanol, 50U/ml penicillin and streptomycin) and cultured in the presence of irradiated (3000rads) P2S#12 pulsed (5mg/ml P2S #12 and 10mg/ml β2-microglobulin)

LPS blasts (A2 transgenic spleens cells cultured in the presence of 7µg/ml dextran sulfate and 25µg/ml LPS for 3 days). Six days later cells (5×10^5 /ml) were restimulated with 2.5×10^6 /ml peptide pulsed irradiated (20,000rads) EL4A2Kb cells (Sherman et al, *Science* 258:815-818, 1992) and 3×10^6 /ml A2 transgenic spleen feeder cells. Cells were cultured in the presence of 20U/ml IL-2. Cells were continued to be restimulated on a weekly basis as mentioned, in preparation for cloning the line.

P2S#12 line was cloned by limiting dilution analysis with peptide pulsed EL4 A2Kb tumor cells (1×10^4 cells/ well) as stimulators and A2 transgenic spleen cells as feeders (5×10^5 cells/ well) grown in the presence of 30U/ml IL-2. On day 14, cells were restimulated as before. On day 21, clones that were growing were isolated and maintained in culture. Several of these clones demonstrated reactivity (lysis) against human fibroblasts (HLA A2.1 expressing) transduced with P502S gene significantly higher than control fibroblasts. An example is presented in Figure 1.

This data indicates that P2S #12 represents a naturally processed epitope of the P502S protein that is expressed in the context of the human HLA A2.1 molecule.

6.2. This Example illustrates the preparation of murine CTL lines and CTL clones specific for cells expressing the P501S gene.

This series of experiments were performed similarly to that described above. Mice were immunized with the P1S #10 peptide (SEQ ID NO: 337), which is derived from the P501S gene (also referred to herein as L1-12, SEQ ID NO:110). The P1S#10 peptide was derived by analysis of the predicted polypeptide sequence for P501S for potential HLA-A2 binding sequences as defined by published HLA-A2 binding motifs (Parker, KC, *et al*, *J. Immunol.*, 152:163, 1994). P1S#10 peptide was synthesized by methods described in Example 4, and empirically tested for HLA-A2 binding using a T cell based competition assay. Predicted A2 binding peptides were tested for their ability to compete HLA-A2 specific peptide presentation to an HLA-A2 restricted CTL clone (D150M58), which is specific for the HLA-A2 binding influenza matrix peptide fluM58. D150M58 CTL secretes TNF in response to self-presentation of peptide fluM58. In the competition assay, test peptides at 100-200 ug/ml were added

to cultures of D150M58 CTL in order to bind HLA-A2 on the CTL. After thirty minutes, CTL cultured with test peptides, or control peptides, were tested for their antigen dose response to the fluM58 peptide in a standard TNF bioassay. Figure 3 shows peptide P1S#10 competes HLA-A2 restricted presentation of fluM58, demonstrating that peptide P1S#10 binds HLA-A2.

Mice expressing the transgene for human HLA A2.1 were immunized as described by Theobald et al., *Proc. Natl. Acad. Sci. USA* 92:11993-11997, 1995 with the following modifications. Mice were immunized with 62.5µg of P1S #10 and 120µg of an I-A^b binding peptide derived from Hepatitis B Virus protein emulsified in incomplete Freund's adjuvant. Three weeks later these mice were sacrificed and using a nylon mesh single cell suspensions prepared. Cells were then resuspended at 6×10^6 cells/ml in complete media (as described above) and cultured in the presence of irradiated (3000rads) P1S#10 pulsed (2µg/ml P1S#10 and 10mg/ml β2-microglobulin) LPS blasts (A2 transgenic spleens cells cultured in the presence of 7µg/ml dextran sulfate and 25µg/ml LPS for 3 days). Six days later cells (5×10^5 /ml) were restimulated with 2.5×10^6 /ml peptide-pulsed irradiated (20,000rads) EL4A2Kb cells, as described above, and 3×10^6 /ml A2 transgenic spleen feeder cells. Cells were cultured in the presence of 20U/ml IL-2. Cells were restimulated on a weekly basis in preparation for cloning. After three rounds of *in vitro* stimulations, one line was generated that recognized P1S#10-pulsed Jurkat A2Kb targets and P501S-transduced Jurkat targets as shown in Figure 4.

A P1S#10-specific CTL line was cloned by limiting dilution analysis with peptide pulsed EL4 A2Kb tumor cells (1×10^4 cells/ well) as stimulators and A2 transgenic spleen cells as feeders (5×10^5 cells/ well) grown in the presence of 30U/ml IL-2. On day 14, cells were restimulated as before. On day 21, viable clones were isolated and maintained in culture. Five of these clones demonstrated specific cytolytic reactivity against P501S-transduced Jurkat A2Kb targets. As shown in Figure 5, this data indicates that P1S#10 represents a naturally processed epitope of the P501S protein that is expressed in the context of the human HLA-A2.1 molecule.

Example 7

ABILITY OF HUMAN T CELLS TO RECOGNIZE PROSTATE TUMOR
POLYPEPTIDES

5

This Example illustrates the ability of T cells specific for a prostate tumor polypeptide to recognize human tumor.

Human CD8⁺ T cells were primed *in vitro* to the P2S-12 peptide (VLGWVAEL; SEQ ID NO:306) derived from the P502S (J1-17) gene using dendritic
 10 cells according to protocol set forth by Van Tsai et al., *Critical Reviews in Immunology* 18:65-75, 1998. The resulting CD8⁺ T cell microcultures were tested for their ability to recognize the P2S-12 peptide presented by autologous fibroblasts or fibroblasts which were transduced to express the P502S gene in a γ -interferon ELISPOT assay (*see* Lalvani et al., *J. Experimental Medicine* 186:859-865, 1997). Briefly, titrating numbers
 15 of T cells were assayed in duplicate on 10⁴ fibroblasts in the presence of 3 μ g/ml human β_2 -microglobulin and 1 μ g/ml P2S-12 peptide or control E75 peptide. In addition, T cells were simultaneously assayed on autologous fibroblasts transduced with the P502S gene or as a control, fibroblasts transduced with HER-2/*neu*. Prior to the assay, the fibroblasts were treated with 10 ng/ml γ -interferon for 48 hours to upregulate class I
 20 MHC expression. One of the microcultures (#5) demonstrated strong recognition of both peptide pulsed fibroblasts as well as transduced fibroblasts in a γ -interferon ELISPOT assay. Figure 2A demonstrates that there was a strong increase in the number of γ -interferon spots with increasing numbers of T cells on fibroblasts pulsed with the P2S-12 peptide (solid bars) but not with the control E75 peptide (open bars). This
 25 shows the ability of these T cells to specifically recognize the P2S-12 peptide. In Figure 2B, this microculture also demonstrated an increase in the number of γ -interferon spots with increasing numbers of T cells on fibroblasts transduced to express the P502S gene but not the HER-2/*neu* gene. These results provide additional confirmatory evidence that the P2S-12 peptide is a naturally processed epitope of the P502S protein.
 30 Furthermore, this also demonstrates that there exists in the human T cell repertoire, high

affinity T cells which are capable of recognizing this epitope. These T cells should also be capable of recognizing human tumors which express the P502S gene.

Example 8

5 PRIMING OF CTL *IN VIVO* USING NAKED DNA IMMUNIZATION WITH A NOVEL PROSTATE ANTIGEN

The novel prostate tumor antigen L1-12, as described above, is also referred to as P501S. HLA A2Kb Tg mice , (provided by Dr L. Sherman, The Scripps Research Institute, La Jolla, CA) were immunized with 100 ug VR10132-P501S either
 10 intramuscularly or intradermally. The mice were immunized three times, with a two week interval between immunizations. Two weeks after the last immunization, immune spleen cells were cultured with Jurkat A2Kb-P501S transduced stimulator cells. CTL lines were stimulated weekly. After two weeks of in vitro stimulation, CTL activity was assessed against P501S transduced targets. The results show that 2/8 mice
 15 developed strong anti-P501S CTL responses. These results demonstrate that P501S contains at least one naturally processed A2-restricted CTL epitope.

Example 9

20 GENERATION OF HUMAN CTL *IN VITRO* USING WHOLE GENE PRIMING AND STIMULATION TECHNIQUES WITH NOVEL PROSTATE TUMOR ANTIGEN

The novel prostate antigen L1-12, as described above, is also referred to
 25 as P501S. Using *in vitro* whole-gene priming with P501S-retrovirally transduced autologous fibroblasts, (see, for example, Yee et al, *The Journal of Immunology*, 157(9):4079-86, 1996) human CTL lines were derived that specifically recognize autologous fibroblasts transduced with P501S, as determined by interferon- γ ELISPOT analysis (as described above). Using a panel of HLA-mismatched fibroblast lines
 30 transduced with P501S, these CTL lines were shown to be restricted HLA-A2 class I

allele. Specifically, dendritic cells (DC) were differentiated from monocyte cultures derived from PBMC of normal human donors by growth for five days in RPMI medium containing 10% human serum and 50 ng/ml human GM-CSF and 30 ng/ml human IL-4. Following culture, DC were infected overnight with recombinant P501S vaccinia virus at a multiplicity of infection (M.O.I) of five, and matured overnight by the addition of 3 µg/ml. CD40 ligand. Virus was inactivated by U.V. irradiation and CD8+ T cells were isolated using a magnetic bead system, and priming cultures were initiated using standard culture techniques. Cultures were restimulated every 7-10 days using autologous primary fibroblasts retrovirally transduced with P501S. Following four stimulations cycles, CD8+ T cell lines could be identified that specifically produced interferon-γ when stimulated with P501S-transduced autologous fibroblasts; the P501S-specific activity could be sustained by the continued stimulation of the cultures with P501S-transduced fibroblasts in the presence of IL-15. A panel of HLA-mismatched fibroblast lines transduced with P501S were generated to define the restriction allele of the response. By measuring Interferon-γ in an ELISPOT assay, the P501S specific response was shown to be restricted by HLA-A2. These results demonstrate that a CD8+ CTL response to P501S can be elicited.

Example 10

IDENTIFICATION OF A NATURALLY PROCESSED CTL EPITOPE CONTAINED WITHIN A NOVEL PROSTATE TUMOR ANTIGEN

The novel prostate antigen P20, as described above, is also referred to as P703P. The 9-mer peptide, p5, having an amino acid sequence of LLANDLMLI, (SEQ ID NO: 338) was derived from the P703P antigen. The p5 peptide is immunogenic in human HLA-A2 donors and is a naturally processed epitope. Antigen specific CD8+ T cells can be primed following repeated *in vitro* stimulations with monocytes pulsed with p5 peptide. These CTL specifically recognize p5-pulsed target cells in both ELISPOT (described above), and chromium release assays. Additionally, in HLA-A2 transgenic mice (described above), immunization with p5 leads to the generation of CTL lines

which recognize a variety of P703P transduced target cells expressing either HLA-A2Kb or HLA-A2. Specifically, HLA-A2 transgenic mice were immunized subcutaneously in the footpad with 100 ug of p5 peptide formulated together with 140 ug of hepatitis B virus core peptide (a Th peptide) in Freund's incomplete adjuvant.

- 5 Three weeks post immunization, spleen cells from immunized mice were stimulated *in vitro* with peptide-pulsed LPS blasts. CTL activity was assessed by chromium release assay five days after primary *in vitro* stimulation. Retrovirally transduced cells expressing P703P, or control antigen, and HLA-A2Kb were used as targets. CTL lines specifically recognized both p5-pulsed targets as well as P703P-expressing targets have
10 been identified.

- Human *in vitro* priming experiments have been conducted that demonstrate the p5 peptide is immunogenic in humans. Dendritic cells (DC) were differentiated from monocyte cultures derived from PBMC of normal human donors by culturing for five days in RPMI medium containing 10% human serum and 50 ng/ml
15 human GM-CSF and 30 ng/ml human IL-4. Following culture, the DC were pulsed with p5 peptide and cultured with GM-CSF and IL-4 together with CD8+ T cell enriched PBMC. CTL lines were restimulated in a weekly basis using p5-pulsed monocytes in subsequent stimulations. Five to six weeks after initiation of the CTL cultures, CTL recognition of p5-pulsed target cells was demonstrated.

20

- From the foregoing, it will be appreciated that, although specific embodiments of the invention have been described herein for the purposes of illustration, various modifications may be made without deviating from the spirit and
25 scope of the invention. Accordingly, the present invention is not limited except as by the appended claims.

CLAIMS

1. A method for determining the presence or absence of prostate cancer in a patient, comprising the steps of:

(a) contacting a biological sample obtained from a patient with a binding agent that binds to a prostate tumor protein, wherein the prostate tumor protein comprises an amino acid sequence that is encoded by a polynucleotide sequence selected from the group consisting of:

(i) polynucleotides recited in any one of SEQ ID NOs:2-3, 5-107, 109-111, 115-171, 173-175, 177, 179-228, 229-305, 307-326, 328, 330, or 332-335; and

(ii) complements of the foregoing polynucleotides; and

(b) detecting in the sample an amount of polypeptide that binds to the binding agent, relative to a predetermined cut-off value, and therefrom determining the presence or absence of a cancer in the patient.

2. A method according to claim 1 wherein the binding agent is a monoclonal antibody.

3. A method according to claim 1 wherein the binding agent is a polyclonal antibody.

4. A method for monitoring the progression of prostate cancer in a patient, comprising the steps of:

(a) contacting a biological sample obtained from a patient at a first point in time with a binding agent that binds to a prostate tumor protein, wherein the prostate tumor protein comprises an amino acid sequence that is encoded by a polynucleotide sequence selected from the group consisting of:

(i) polynucleotides recited in any one of SEQ ID NOs:2-3, 5-107, 109-111, 115-171, 173-175, 177, 179-228, 229-305, 307-326, 328, 330, or 332-335; and

(ii) complements of the foregoing polynucleotides;

(b) detecting in the sample an amount of polypeptide that binds to the binding agent;

(c) repeating steps (a) and (b) using a biological sample obtained from the patient at a subsequent point in time; and

(d) comparing the amount of polypeptide detected in step (c) to the amount detected in step (b) and therefrom monitoring the progression of the cancer in the patient.

5. A method according to claim 4 wherein the binding agent is a monoclonal antibody.

6. A method according to claim 4 wherein the binding agent is a polyclonal antibody.

7. A method for determining the presence or absence of prostate cancer in a patient, comprising the steps of:

(a) contacting a biological sample obtained from a patient with an oligonucleotide that hybridizes to a polynucleotide that encodes a prostate tumor protein, wherein the prostate tumor protein comprises an amino acid sequence that is encoded by a polynucleotide sequence selected from the group consisting of:

(i) polynucleotides recited in any one of SEQ ID NOs:2-3, 5-107, 109-111, 115-171, 173-175, 177, 179-228, 229-305, 307-326, 328, 330, or 332-335; and

(ii) complements of the foregoing polynucleotides; and

(b) detecting in the sample an amount of a polynucleotide that hybridizes to the oligonucleotide, relative to a predetermined cut-off value, and therefrom determining the presence or absence of a cancer in the patient.

8. A method according to claim 7, wherein the amount of polynucleotide that hybridizes to the oligonucleotide is determined using polymerase chain reaction.

9. A method according to claim 7, wherein the amount of polynucleotide that hybridizes to the oligonucleotide is determined using a hybridization assay.

10. A method for monitoring the progression of a cancer in a patient, comprising the steps of:

(a) contacting a biological sample obtained from a patient with an oligonucleotide that hybridizes to a polynucleotide that encodes a prostate tumor protein, wherein the prostate tumor protein comprises an amino acid sequence that is encoded by a polynucleotide sequence selected from the group consisting of:

(i) polynucleotides recited in any one of SEQ ID NOs:2-3, 5-107, 109-111, 115-171, 173-175, 177, 179-228, 229-305, 307-326, 328, 330, or 332-335; and

(ii) complements of the foregoing polynucleotides;

(b) detecting in the sample an amount of a polynucleotide that hybridizes to the oligonucleotide;

(c) repeating steps (a) and (b) using a biological sample obtained from the patient at a subsequent point in time; and

(d) comparing the amount of polynucleotide detected in step (c) to the amount detected in step (b) and therefrom monitoring the progression of the cancer in the patient.

11. A method according to claim 10, wherein the amount of polynucleotide that hybridizes to the oligonucleotide is determined using polymerase chain reaction.

12. A method according to claim 10, wherein the amount of polynucleotide that hybridizes to the oligonucleotide is determined using a hybridization assay.

13. An isolated antibody that specifically binds to a prostate tumor protein, wherein the prostate tumor protein comprises an amino acid sequence that is encoded by a polynucleotide sequence selected from the group consisting of:

(i) polynucleotides recited in any one of SEQ ID NOS: 2-3, 8-29, 41-45, 47-52, 54-65, 70, 73, 74, 79, 81, 87, 90, 92, 93, 97, 103, 104, 107, 109-111, 115-160, 171, 173-175, 177, 181, 188, 191, 193, 194, 198, 203, 204, 207, 209, 220, 222-225, 227-305, 307-315, 326, 328, 330, 332, or 334; and

(ii) complements of the foregoing polynucleotides.

14. An antibody according to claim 13, wherein the antibody is a monoclonal antibody.

15. A diagnostic kit comprising:

- (a) one or more monoclonal antibodies according to claim 14; and
- (b) a detection reagent.

16. A diagnostic kit comprising:

(a) one or more monoclonal antibodies that specifically bind to a prostate tumor protein, wherein the prostate tumor protein comprises an amino acid sequence that is encoded by a polynucleotide sequence selected from the group consisting of:

(i) polynucleotides recited in any one of SEQ ID NOS: 5-7, 30-40, 46, 53, 66-69, 71, 72, 75-78, 80, 82-86, 88, 89, 91, 94-96, 98-102, 105, 106, 161-170, 179, 180, 182-187, 189, 190, 192, 195-197, 199-202, 205, 206, 225, 227, 228, 229-305, 316-325, 333, and 335; and

(ii) complements of the foregoing polynucleotides; and

- (b) a detection reagent.

17. A kit according to claim 15 or claim 16 wherein the monoclonal antibodies are immobilized on a solid support.

18. A kit according to claim 17 wherein the solid support comprises nitrocellulose, latex or a plastic material.

19. A kit according to claim 15 or claim 16 wherein the detection reagent comprises a reporter group.

20. The kit of claim 14 wherein the detection reagent comprises an anti-immunoglobulin, Protein G, Protein A or lectin.

21. A kit according to claim 19 wherein the reporter group is selected from the group consisting of radioisotopes, fluorescent groups, luminescent groups, enzymes, biotin and dye particles.

22. An oligonucleotide comprising 10 to 40 nucleotides that hybridize under moderately stringent conditions to a polynucleotide that encodes a prostate tumor protein, wherein the prostate tumor protein comprises an amino acid sequence that is encoded by a polynucleotide sequence selected from the group consisting of:

- (i) polynucleotides recited in any one of SEQ ID NOs:2-3, 5-107, 109-111, 115-171, 173-175, 177, 179-228, 229-305, 307-326, 328, 330, or 332-335; and
- (ii) complements of the foregoing polynucleotides.

23. A oligonucleotide according to claim 22, wherein the oligonucleotide comprises 10-40 nucleotides recited within any one of SEQ ID NOs:2-3, 5-107, 109-111, 115-171, 173-175, 177, 179-228, 229-305, 307-326, 328, 330, or 332-335.

24. A diagnostic kit, comprising:

- (a) an oligonucleotide according to claim 22; and
- (b) a diagnostic reagent for use in a polymerase chain reaction or hybridization assay.

25. A diagnostic kit, comprising:

- (a) an oligonucleotide according to claim 22; and
- (b) a second oligonucleotide 10-40 nucleotides in length.

COMPOUNDS FOR IMMUNODIAGNOSIS OF PROSTATE CANCER
AND METHODS FOR THEIR USE

ABSTRACT OF THE DISCLOSURE

Compounds and methods for diagnosing prostate cancer are provided. The inventive compounds include polypeptides containing at least a portion of a prostate tumor protein. The inventive polypeptides may be used to generate antibodies useful for the diagnosis and monitoring of prostate cancer. Nucleic acid sequences for preparing probes, primers, and polypeptides are also provided.

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<210> 325

<211> 400

<212> DNA

<213> Human

<400> 325

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<211> 1215

<212> DNA

<213> Human

<400> 326

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<210> 327
 <211> 220
 <212> PRT
 <213> Human

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<400> 327
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Leu Ser Ala Ala His Cys Phe Gln Asn Ser Tyr Thr Ile Gly Leu Gly
35     40     45
Leu His Ser Leu Glu Ala Asp Gln Glu Pro Gly Ser Gln Met Val Glu
50     55     60
Ala Ser Leu Ser Val Arg His Pro Glu Tyr Asn Arg Pro Leu Leu Ala
65     70     75     80
Asn Asp Leu Met Leu Ile Lys Leu Asp Glu Ser Val Ser Glu Ser Asp
85     90     95
Thr Ile Arg Ser Ile Ser Ile Ala Ser Gln Cys Pro Thr Ala Gly Asn
100    105    110
Ser Cys Leu Val Ser Gly Trp Gly Leu Leu Ala Asn Gly Arg Met Pro
115    120    125
Thr Val Leu Gln Cys Val Asn Val Ser Val Val Ser Glu Glu Val Cys
130    135    140
Ser Lys Leu Tyr Asp Pro Leu Tyr His Pro Ser Met Phe Cys Ala Gly
145    150    155    160
Gly Gly Gln Asp Gln Lys Asp Ser Cys Asn Gly Asp Ser Gly Gly Pro
165    170    175
Leu Ile Cys Asn Gly Tyr Leu Gln Gly Leu Val Ser Phe Gly Lys Ala
180    185    190
Pro Cys Gly Gln Val Gly Val Pro Gly Val Tyr Thr Asn Leu Cys Lys
195    200    205
Phe Thr Glu Trp Ile Glu Lys Thr Val Gln Ala Ser
210    215    220

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<210> 328
 <211> 234
 <212> DNA
 <213> Human

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atccgcagtg ggtgctgtca gccacacact gtttccagaa ctctacacc atcgggctgg    180
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<210> 329

<211> 77
 <212> PRT
 <213> Human

<400> 329
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 35 40 45
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 50 55 60
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 65 70 75

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<210> 331
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 <212> PRT
 <213> Human

<400> 331
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 Val Ser Gly Ser Cys Ser
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 <212> DNA
 <213> Human

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<210> 333

<211> 3030

<212> DNA

<213> Human

<400> 333

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<210> 334

<211> 2417

<212> DNA

<213> Human

<400> 334

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<211> 2984

<212> DNA

<213> Human

<400> 335

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35          40          45
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50          55          60
Pro Glu Arg Ala His Leu Ala Lys Asn Leu Lys Leu Thr Glu Thr Gln
65          70          75          80
Val Lys Ile Trp Phe Gln Asn Arg Arg Tyr Lys Thr Lys Arg Lys Gln
85          90          95
Leu Ser Ser Glu Leu Gly Asp Leu Glu Lys His Ser Ser Leu Pro Ala
100         105         110
Leu Lys Glu Glu Ala Phe Ser Arg Ala Ser Leu Val Ser Val Tyr Asn
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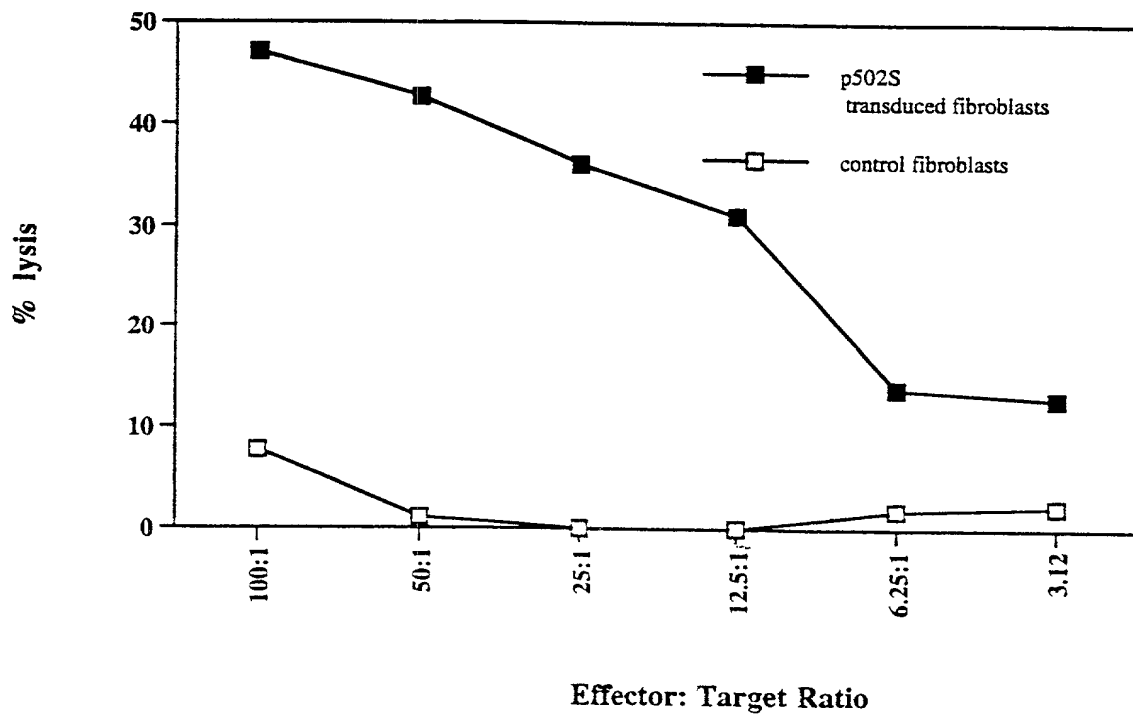


FIG. 1

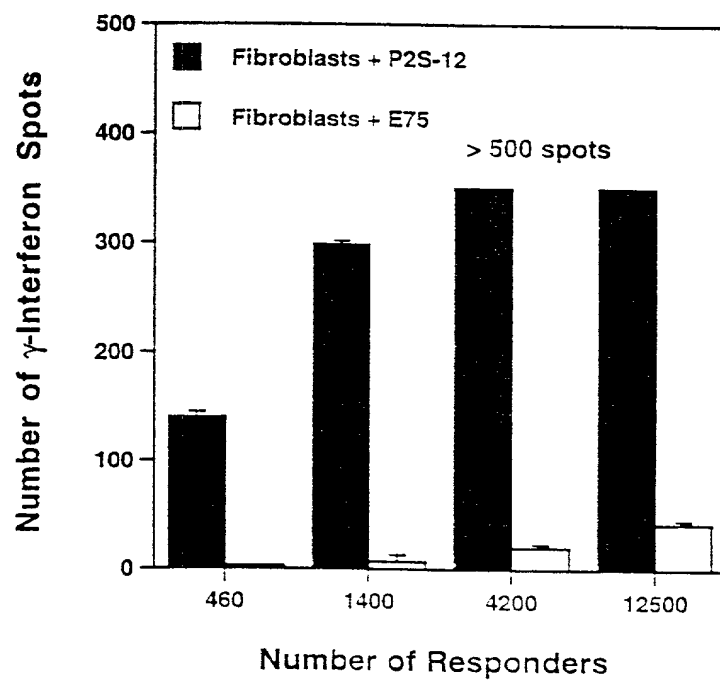


FIG. 2A

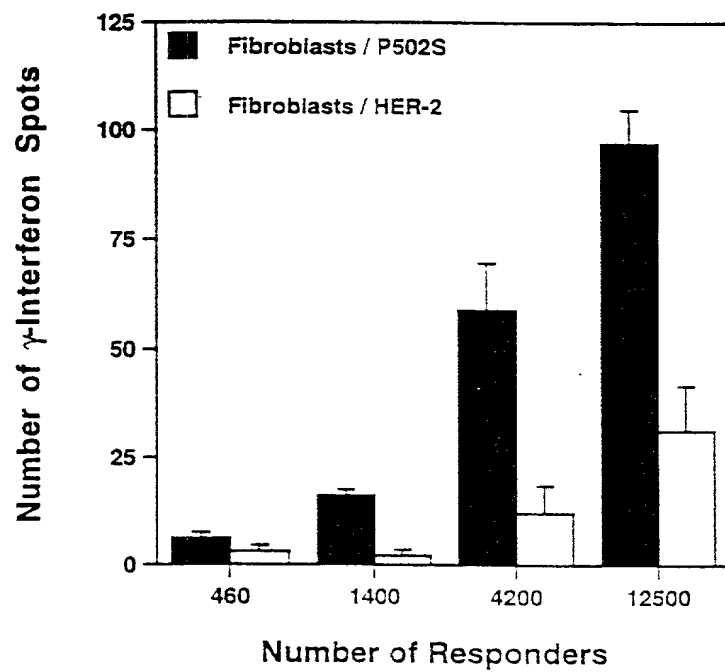
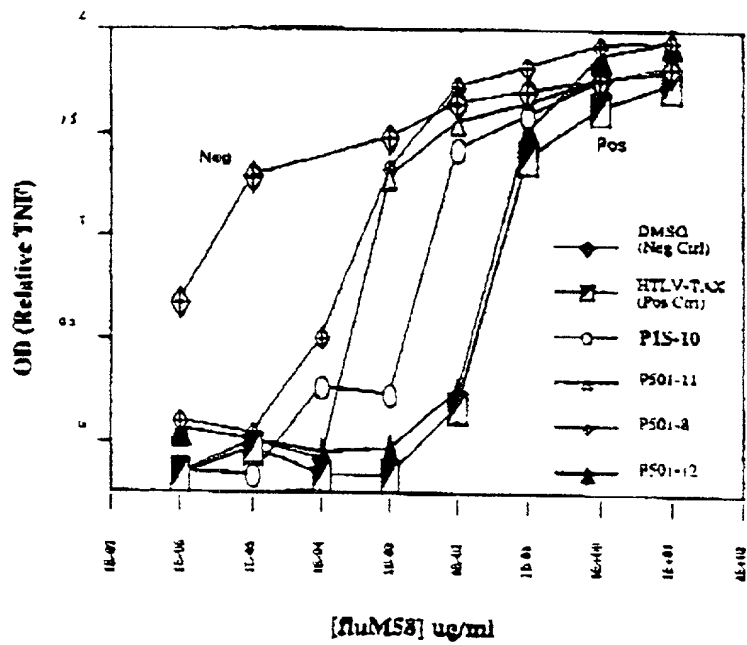


FIG. 2B



Figure

3

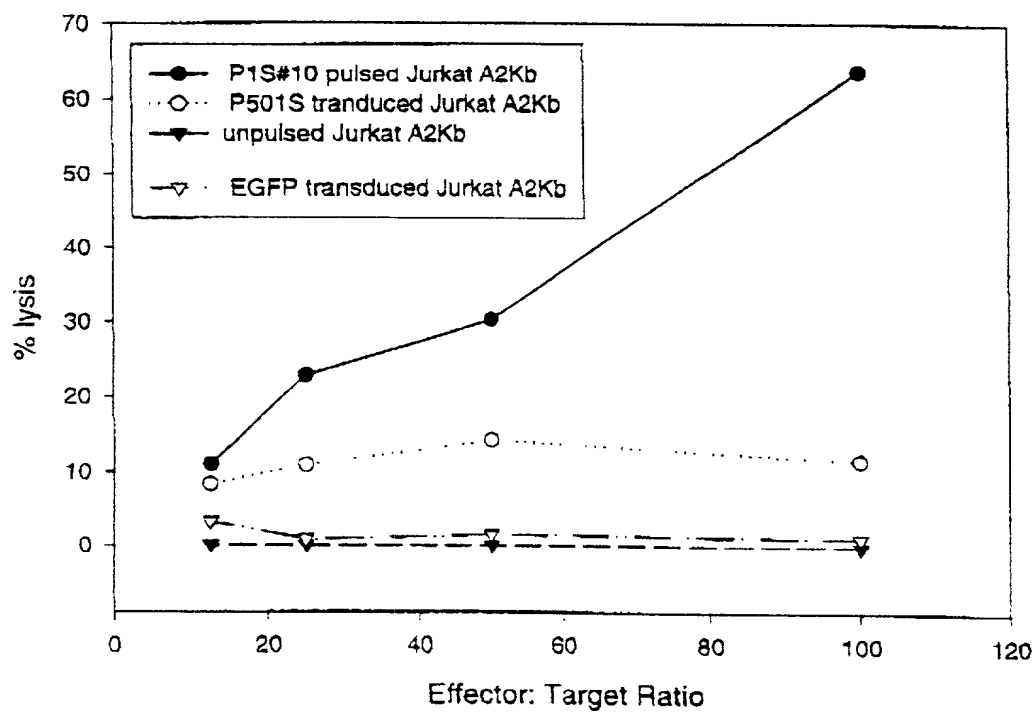


Figure 4

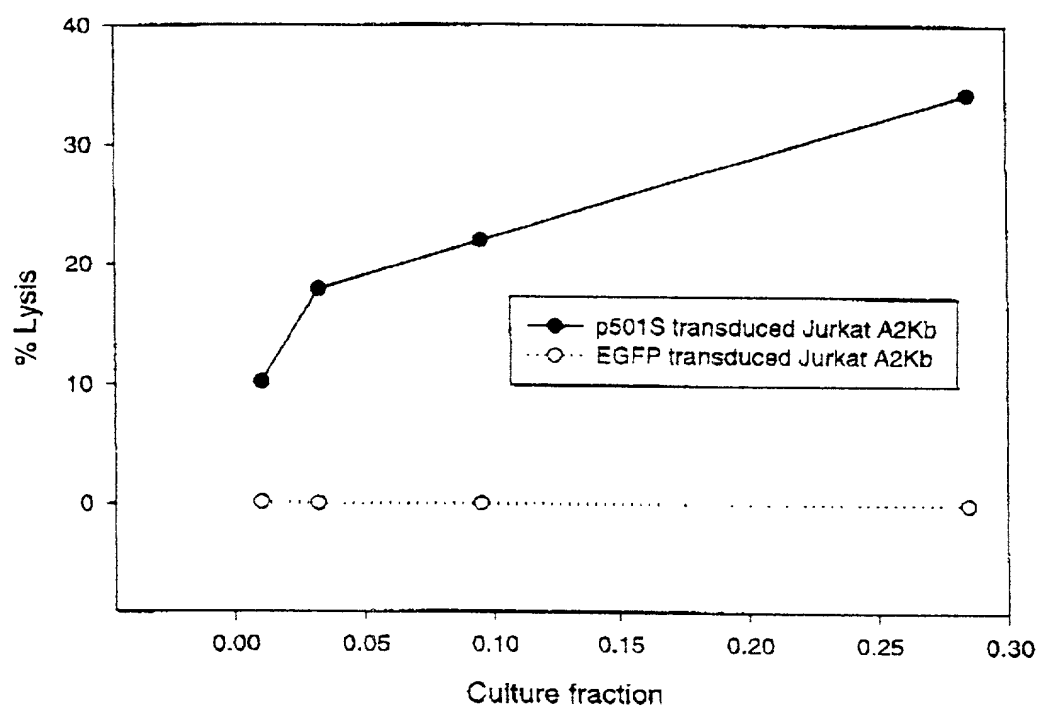


Figure 5